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Pitfalls of sc/snRNA-seq

October 30, 2025

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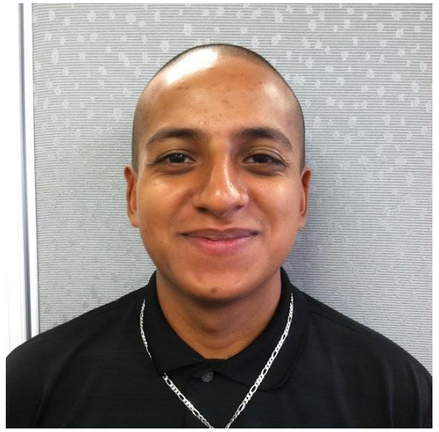
St. Jude Children's Research Hospital

The DNB Bioinformatics Core Team



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Pitfalls of sc/snRNA-seq workshop outline

- Purpose: highlight analysis steps where pitfalls occur
- Explore, fine tune, especially if data returns unexpected results
- Going to cover:
  - Cell Level QC and Filtering
  - Normalization, Variable Feature Selection, Scaling, Regression
  - Dimensionality Reduction, Clustering, Visualization
  - Integration
  - Differential Expression Analysis
  - Cell Type Annotation
- Focused on analysis in Seurat, but pitfalls are general to any sc/snRNAseq analysis

Pre-Seurat QC Steps

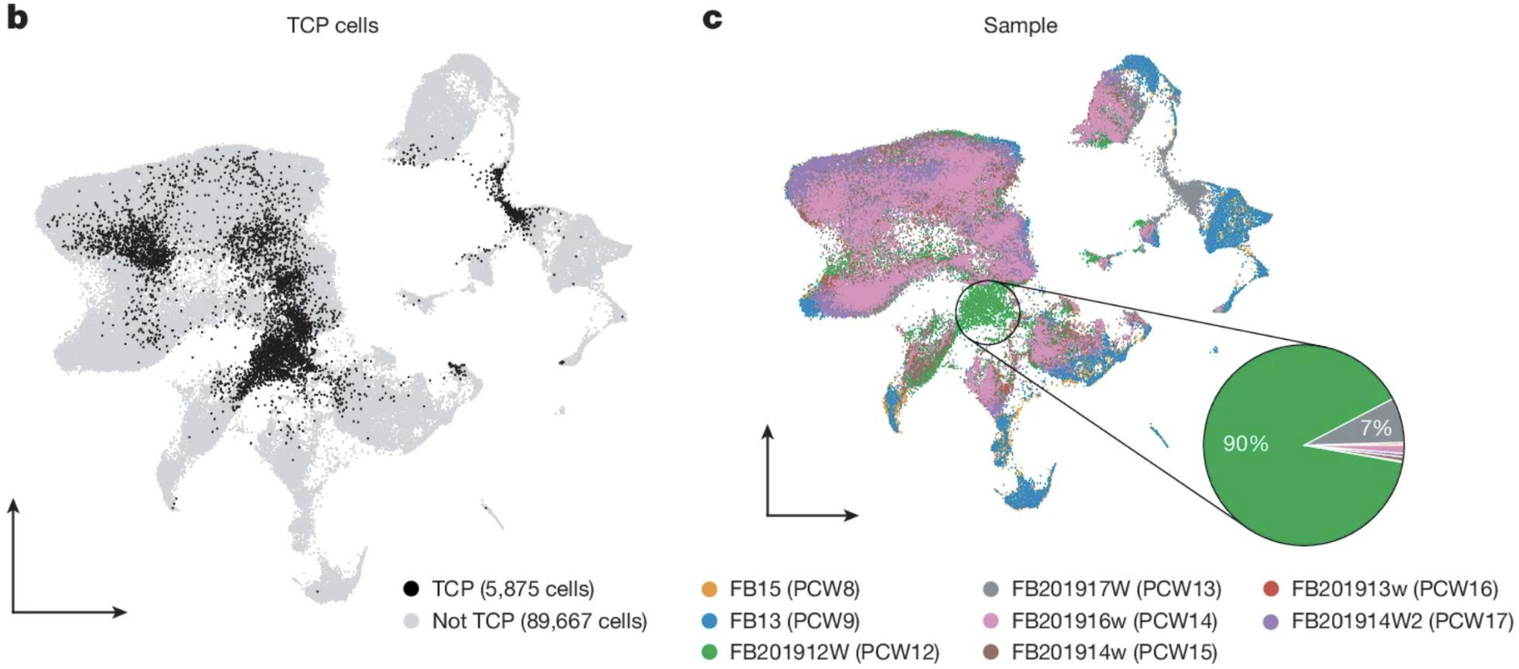
- Look at quality of raw sequencing with fastqs
  - Run FastQC, review sequencing metrics
- Look at quality of alignment
  - Review mapping quality, number of cells, genes, etc.
- See DNB Bioinformatics Core trainings for additional info

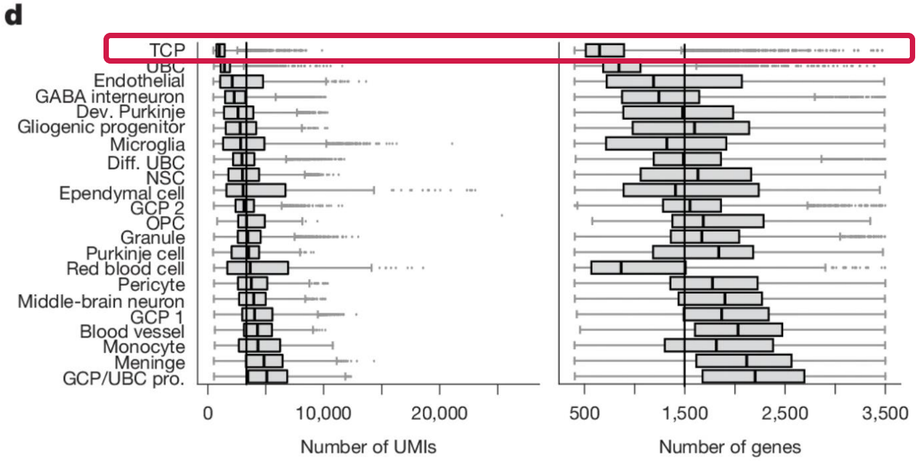
Intro to sc/snRNA-seq workshop

Cell Level QC and Filtering

- First step after alignment, always filter cells
- Low quality cells not informative of true biology
- Skews results, interpretation, can lead to false conclusions
- Cutoffs depend on specific project, data type, sequencing depth
- Choosing appropriate cutoffs is important
    - Too low, keep low quality cells
    - Too high, lose informative cells

Cell Level QC and Filtering



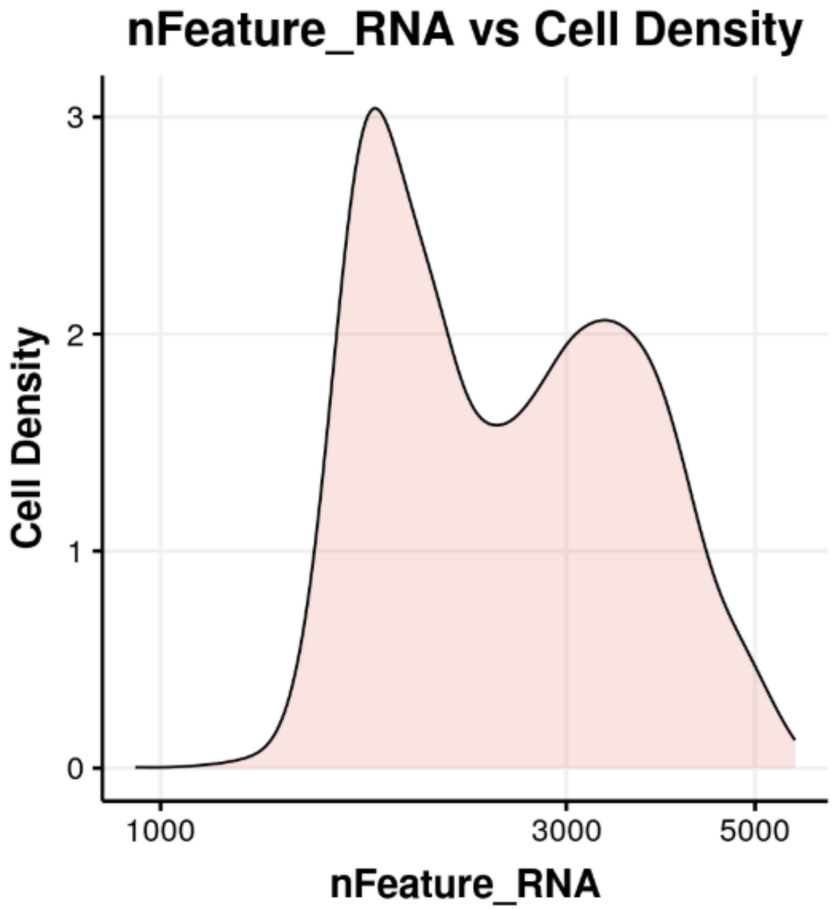


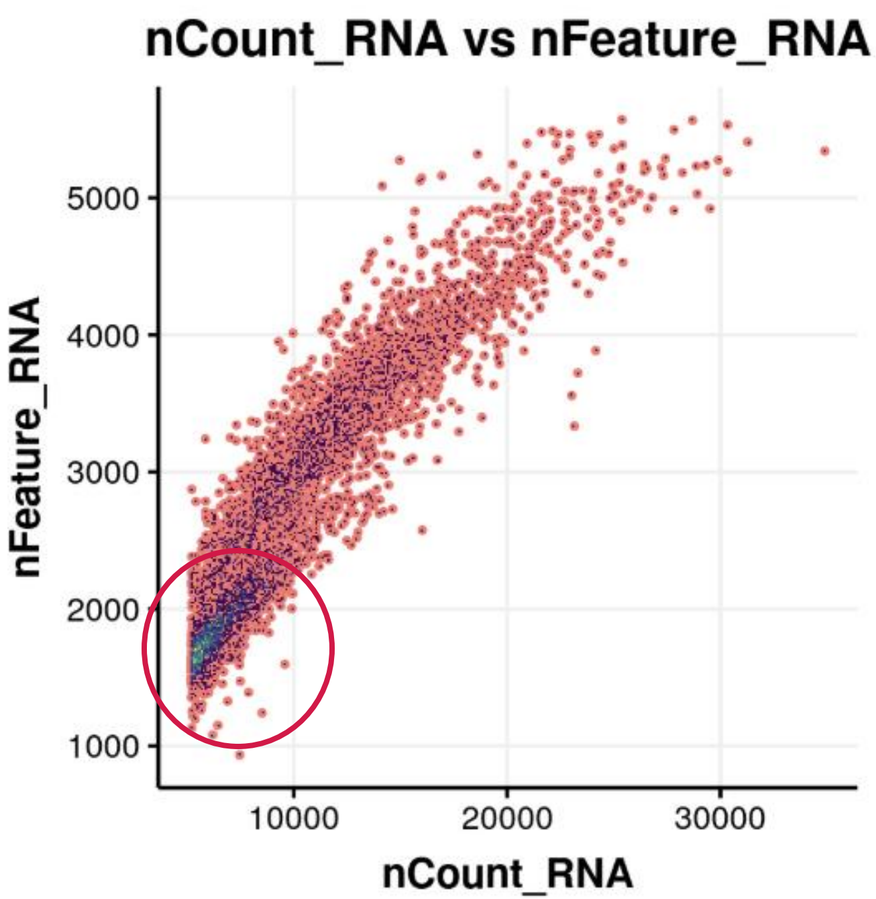
Smith (2025). Nature.

Cell Level QC and Filtering

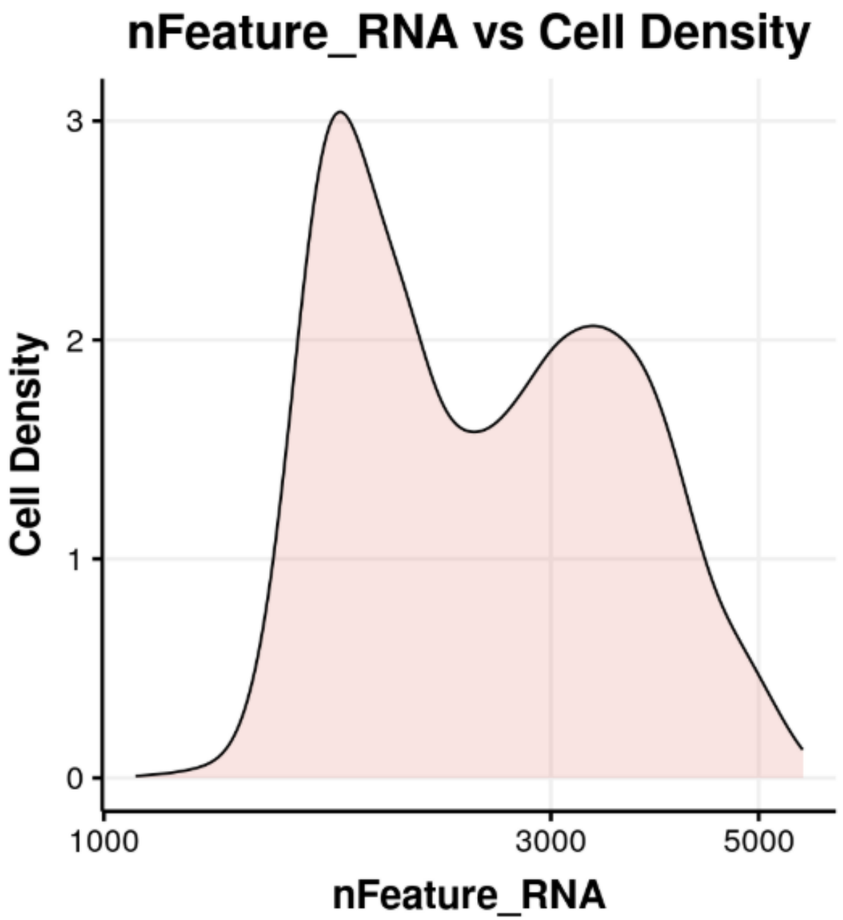
- Minimum: at least 300 genes, at least 500 UMI, less than 5% (scRNA-seq) or 1% (snRNA-seq) mitochondrial expression
- Consider applying outlier filters, e.g. median absolute deviation (MAD)
- Apply filtering to samples individually
- Test multiple parameter sets
- Search literature for cutoffs used for similar cell types, models
- Look at distributions, other plots of metrics
    - Bimodal distributions, indications of lower diversity, cells grouping on filtering metrics in UMAP

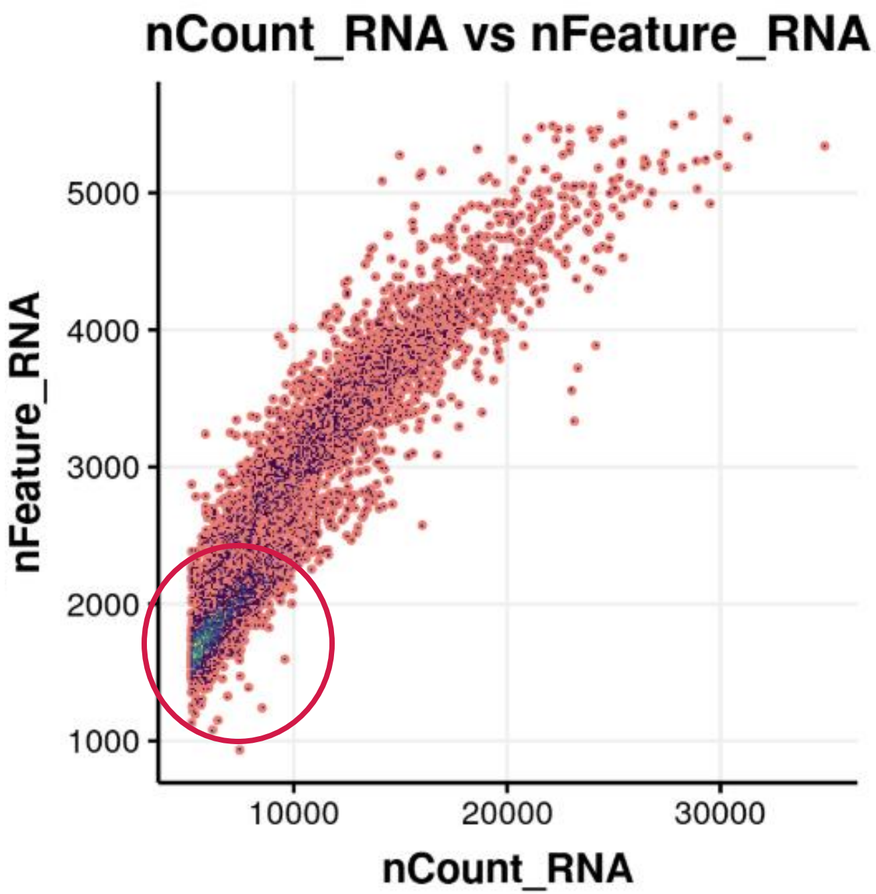
Example: 300 gene + 500 UMI, n cells = 4,837



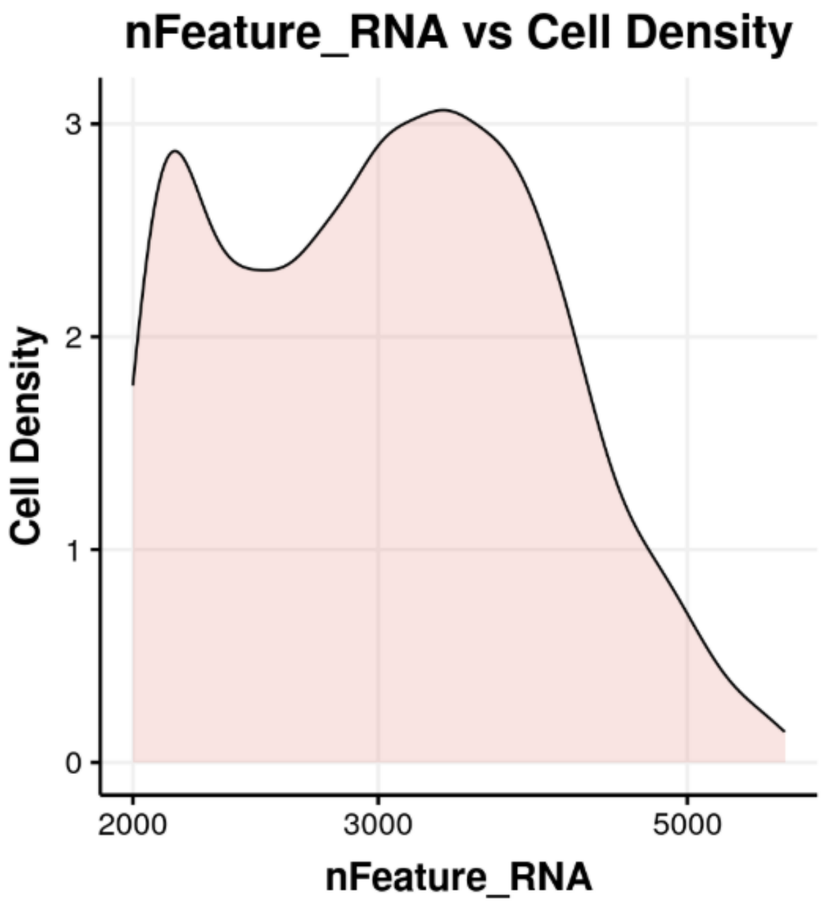


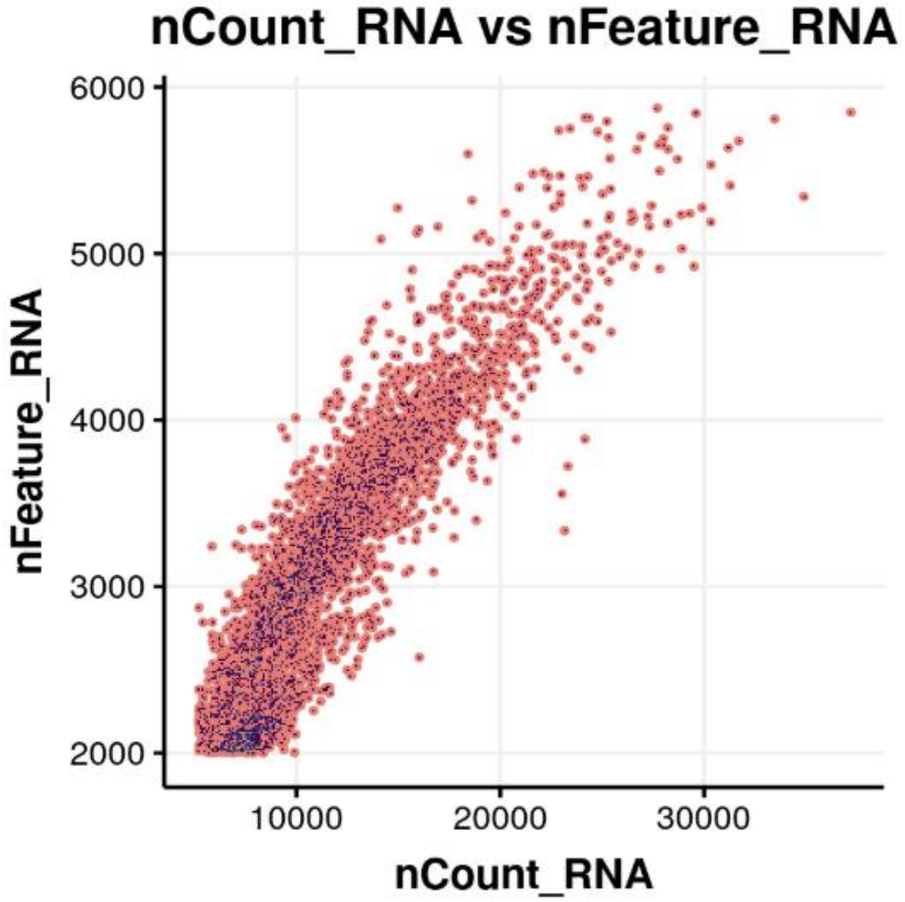
1k gene + 2k UMI, n cells = 4,836





2k gene + 3k UMI, n cells = 3,279

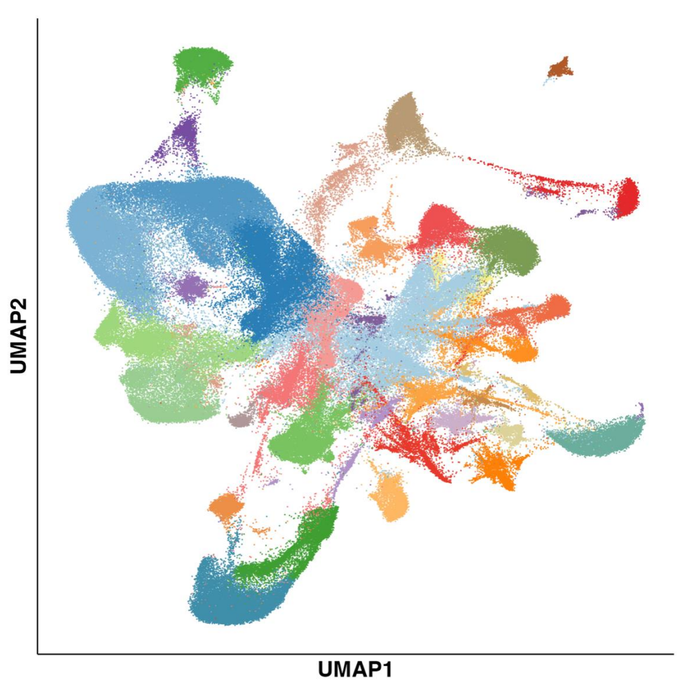


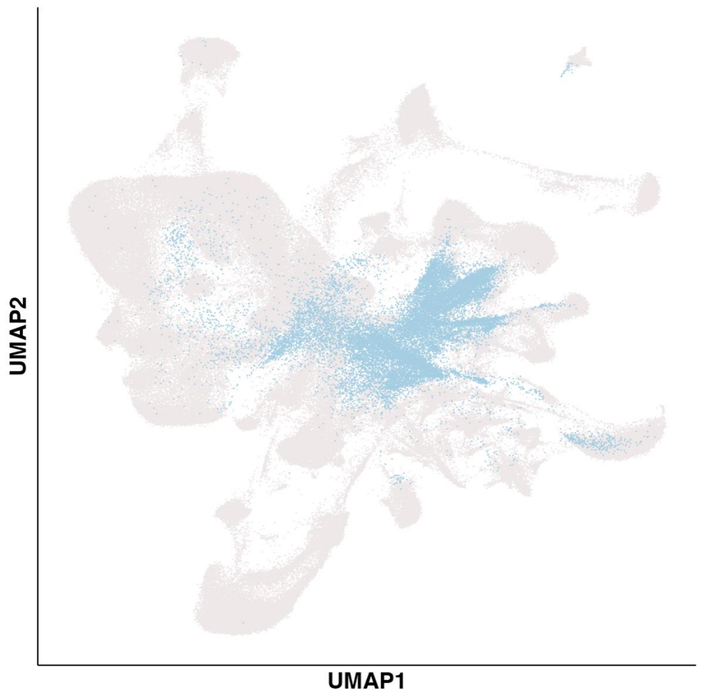


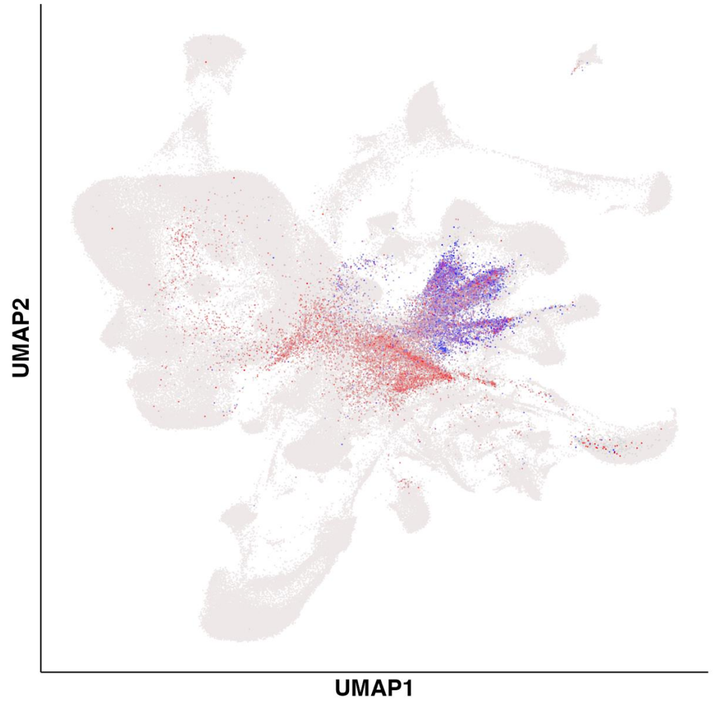
Cell Level QC and Filtering

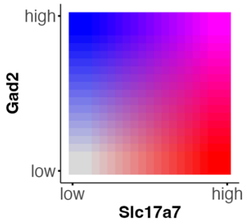
- May need to correct for ambient RNA contamination
- May need to filter out doublets
- Do not recommend correcting, filtering initially
- Tools can struggle with differentiating cell types, overlapping expression
- Patterns in UMAPs, expression could indicate contamination, doublets
    - Spiderwebbing (ambient RNA contamination)
    - Lack of distinct clusters
    - Overlap in mutually exclusive marker expression

Cell Level QC and Filtering

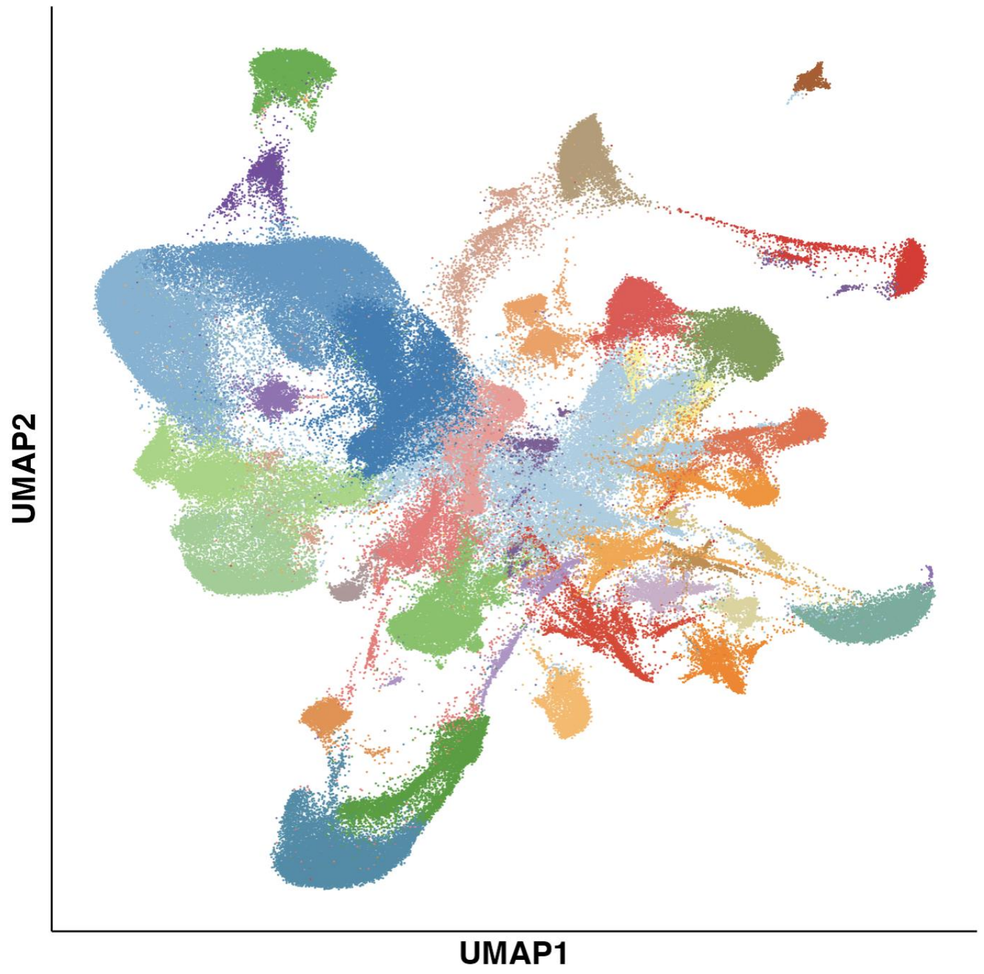


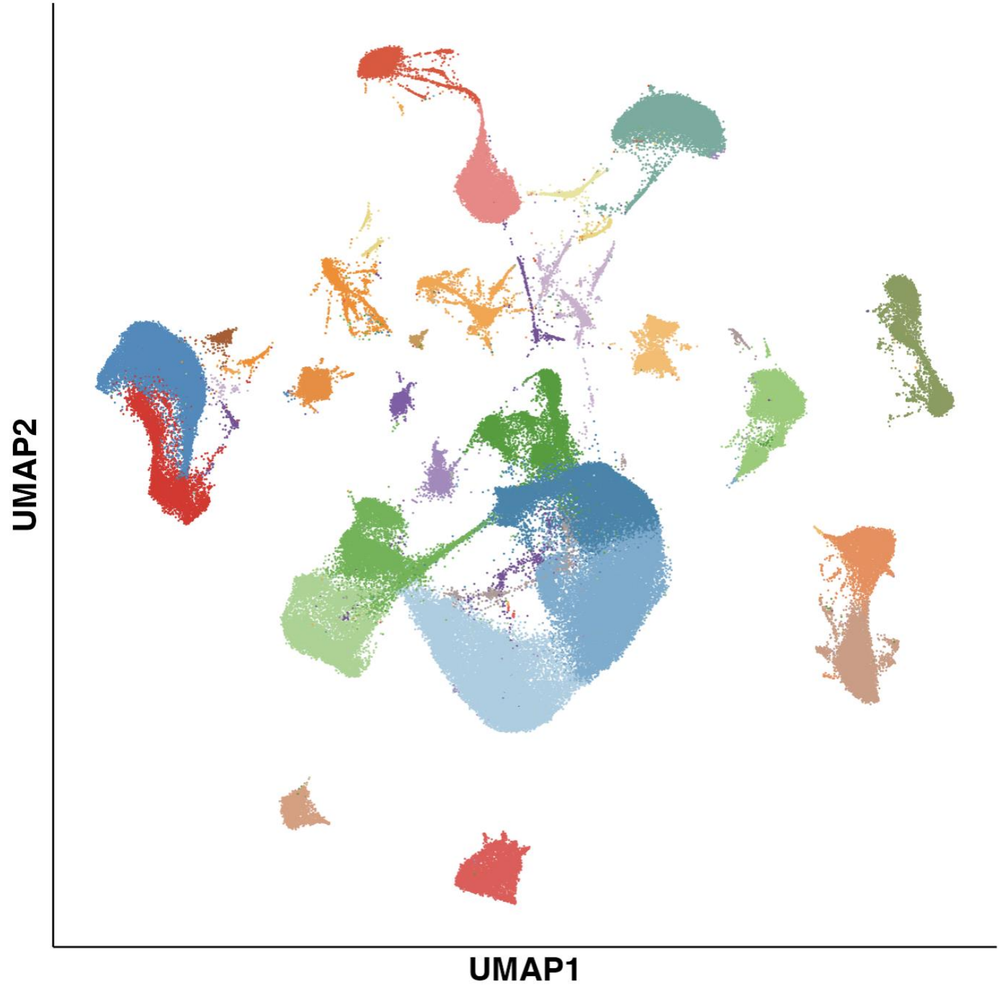






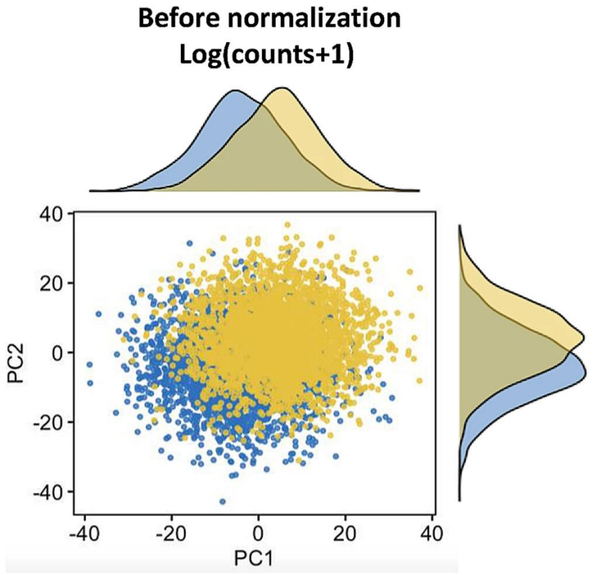
Cell Level QC and Filtering

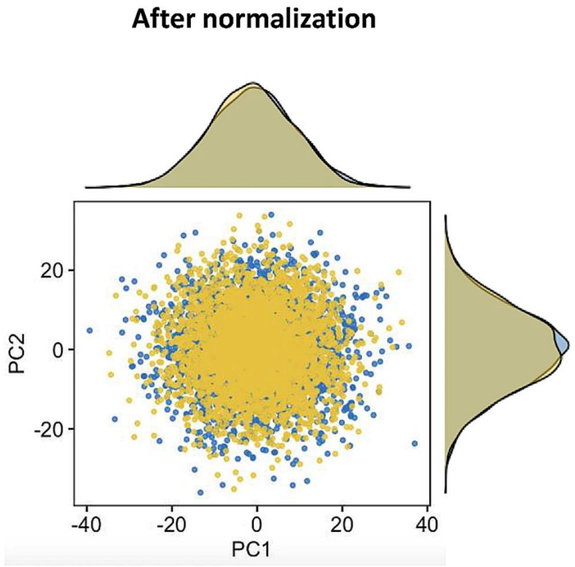




Normalization

- Important to make gene counts comparable
  - Relevant within and across samples
  - Reduces sources of technical noise
- Seurat has several methods:
  - NormalizeData()
    - LogNormalize (default)
    - Relative Counts
    - Center Log Ratio Transformation
  - SCTransform()
- Must apply the same method across samples





Cuevas-Diaz Duran (2024). BMC Genomics.

Highly Variable Gene (HVG) Selection

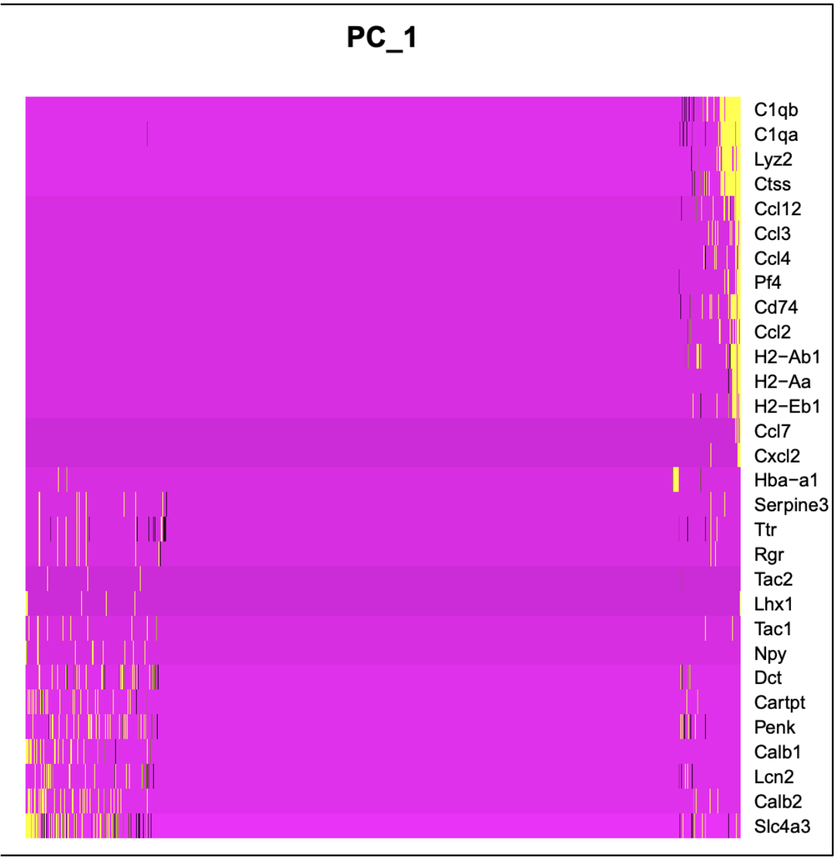
- Identify genes highly expressed in some cells, lowly expressed in others
- HVGs impact downstream results
  - Used for principal component analysis (PCA)
  - PCs used for clustering, non-linear dimensionality reduction, etc.
- Focus on HVGs shown to highlight biological signals
- Feature selection method is important
  - Variance alone may not reflect biology
  - Consider mean-variance relationship

Highly Variable Gene (HVG) Selection

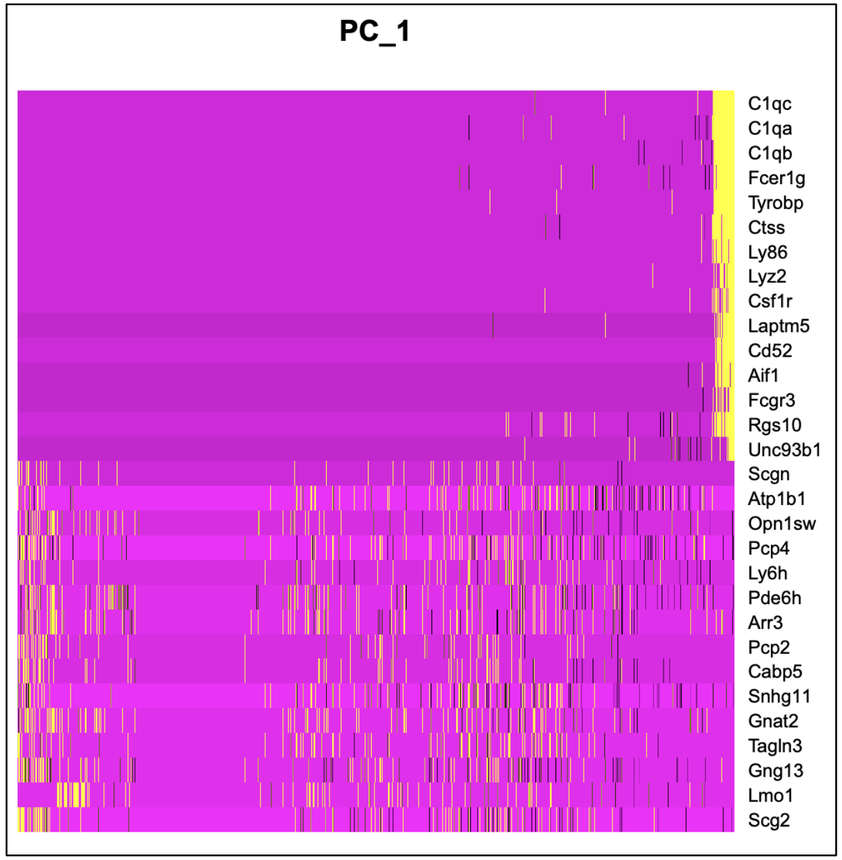
- Seurat default is 2000
- DNB Bioinformatics core default typically 3000
- Won’t necessarily change this parameter
- Be aware number of HVGs impacts downstream results
- Always rerun after merging multiple samples

Highly Variable Gene (HVG) Selection

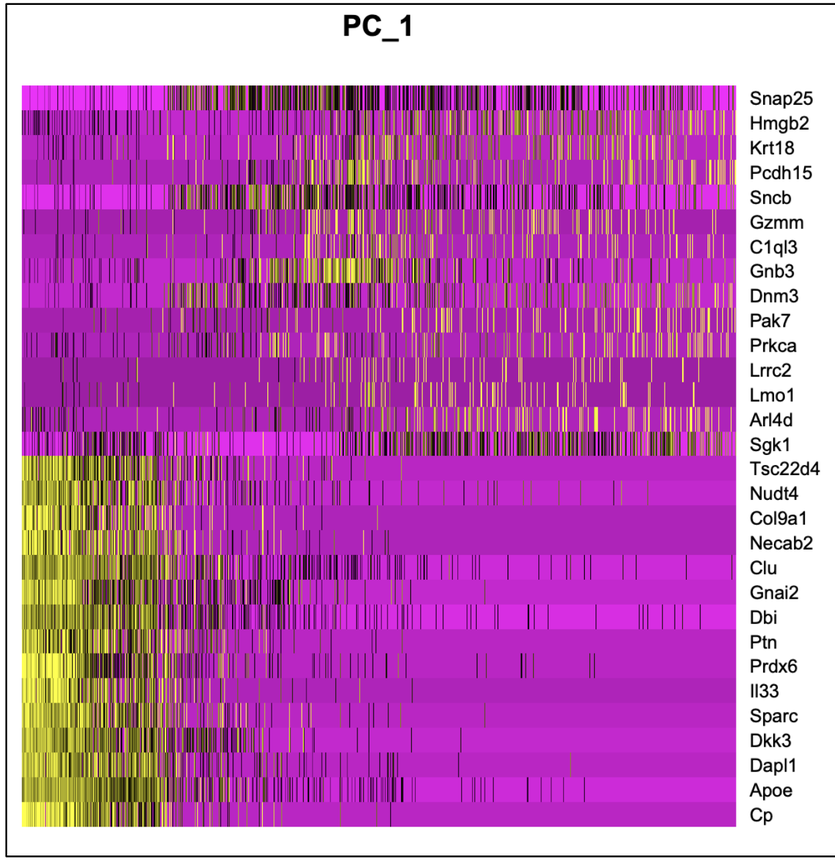
of HVGs = 30



of HVGs = 300

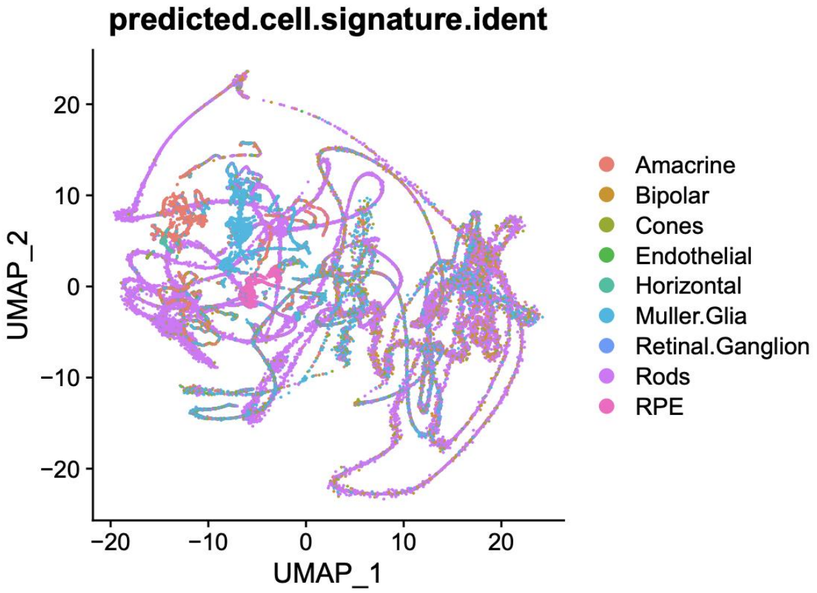


of HVGs = 3000

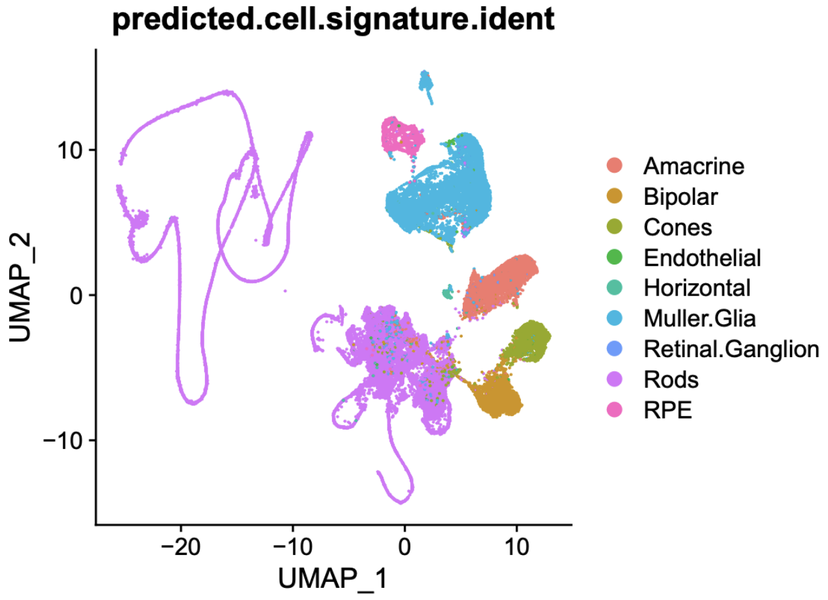


Highly Variable Gene (HVG) Selection

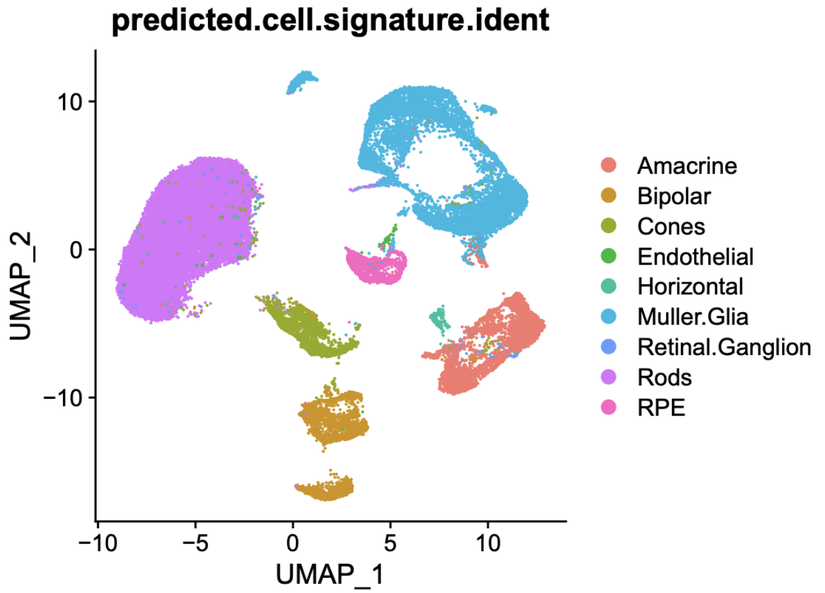
of HVGs = 30



of HVGs = 300



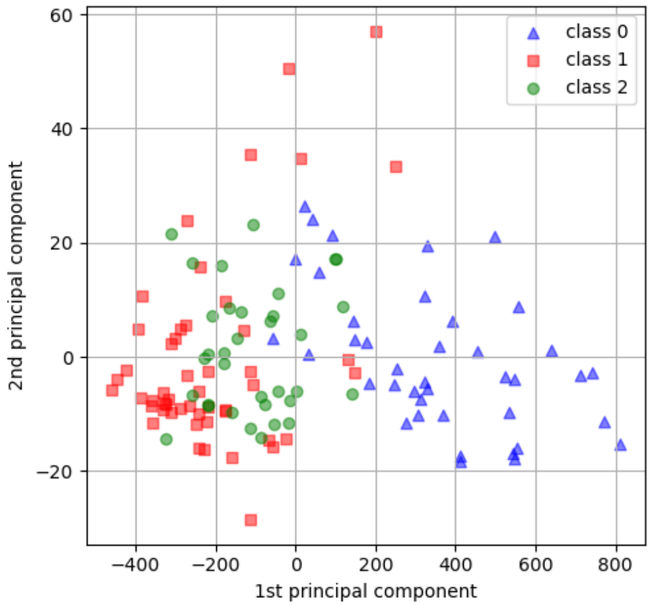
of HVGs = 3000



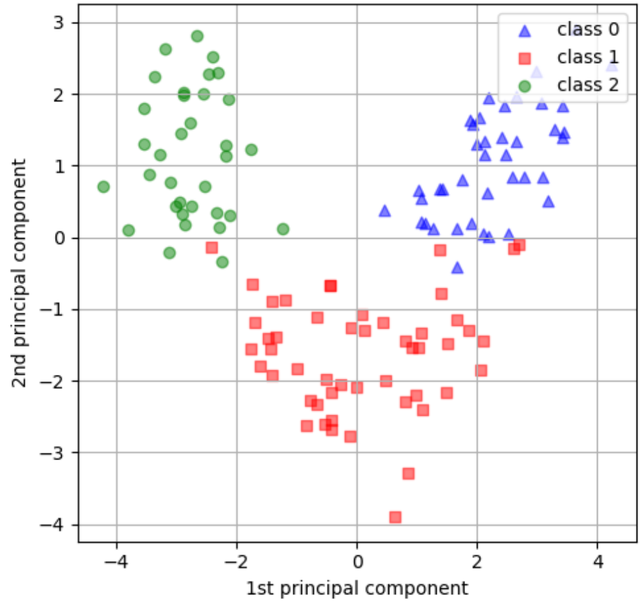
Scaling

- Scaling performed per gene
- Makes gene expression more comparable across cells, samples
- Reduces dominance of highly expressed genes
- Important for principal component analysis, downstream steps

PCA on unscaled data



PCA on scaled data



[Importance of Feature Scaling.]

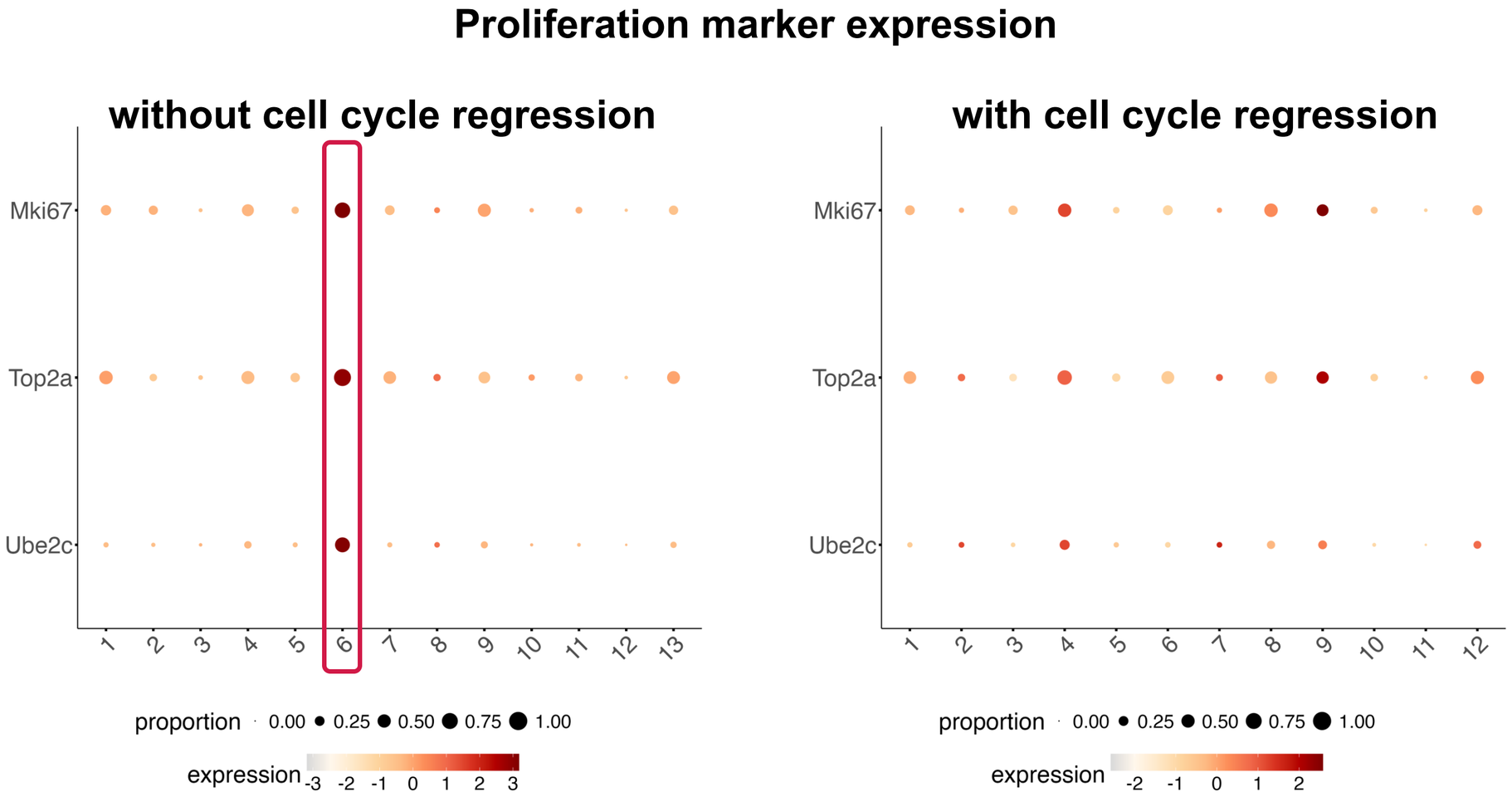
Scaling

- Center and scale expression of each gene
  - Mean = 0
  - Standard deviation = 1
  - z-score transformation
- Scale before running PCA
- Always scaling rerun after merging samples
- Not rescaling can contribute to batch effects, technical artefacts

Scaling and Regression

- Can do regression during scaling step
- Regress out technical or biological variables
  - Cell cycle genes, mitochondrial genes, ribosomal genes, UMI count, batch
- Do analysis without regression first, add regression later if needed
- Seurat has three models for regression
  - Linear (default), Poisson (GLM), Negative Binomial (GLM)
- Regression can have unintended effects
- If regressing multiple variables, regress in same step

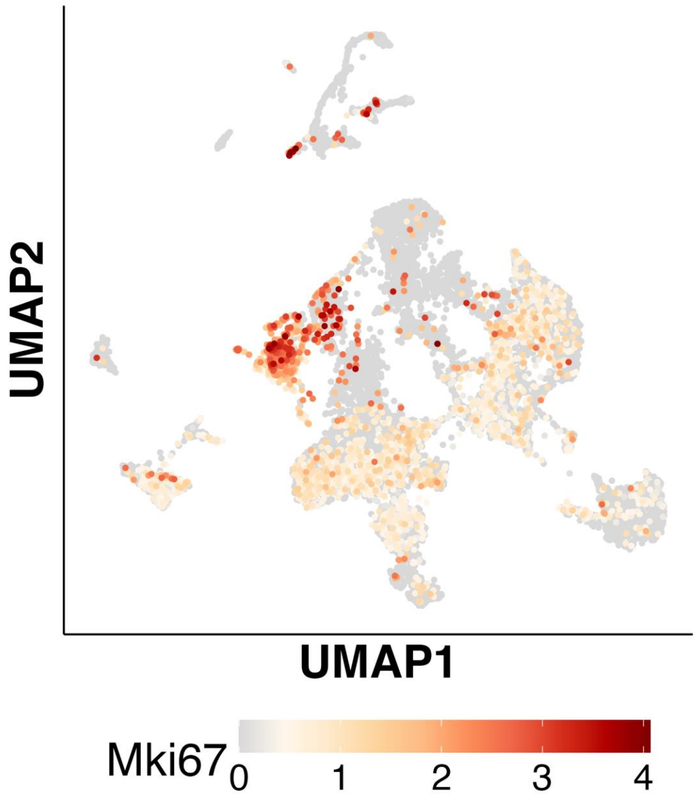
Scaling and Regression



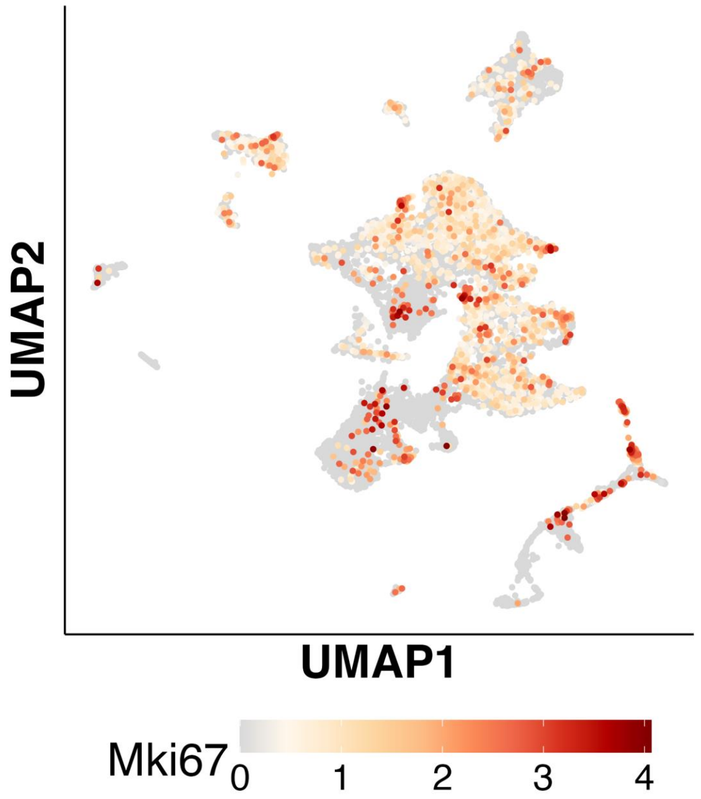
Scaling and Regression

Proliferation marker expression

without cell cycle regression



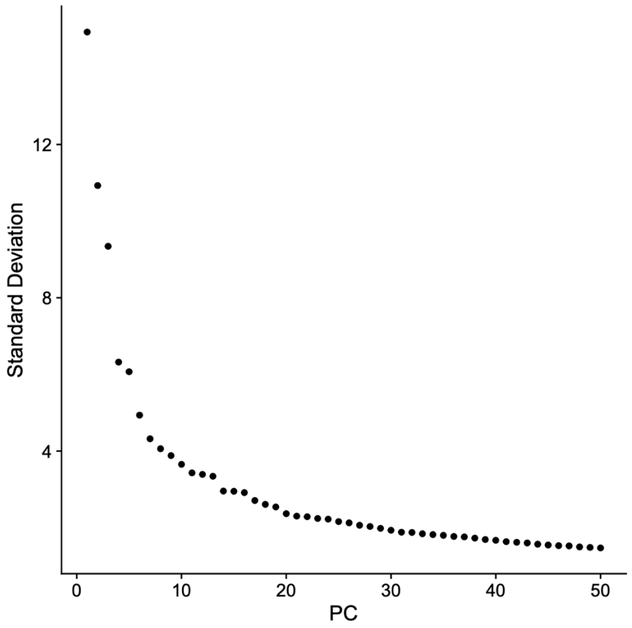
with cell cycle regression



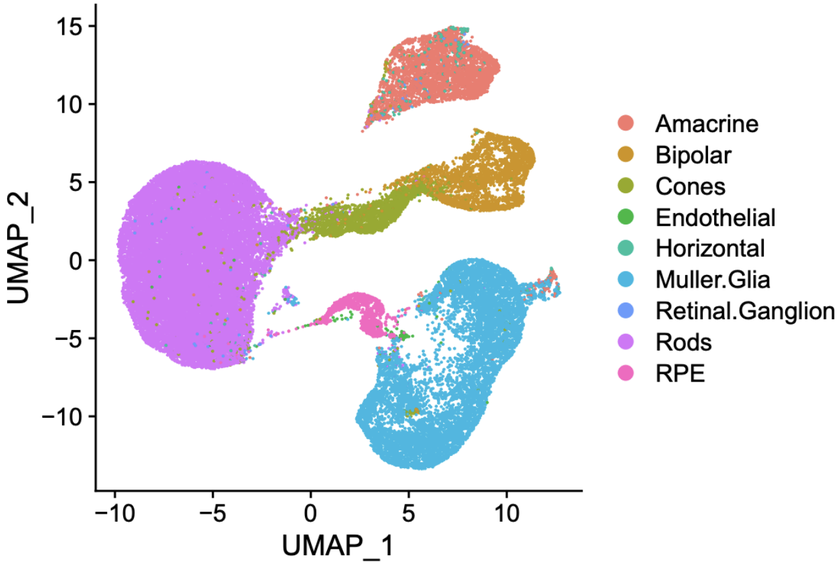
PCA (linear dimensionality reduction)

- Principal component analysis (PCA) is common linear dimensionality reduction
  - Identify gene sets with correlated expression patterns
  - Capture heterogeneity across cells with fewer dimensions
- Choose an appropriate number of PCs
  - Too few, won’t distinguish biology
  - Too many, include technical noise, have diminishing returns
- Sufficient range typically 10 to 50 PCs
- Use elbow plots for choosing reasonable number

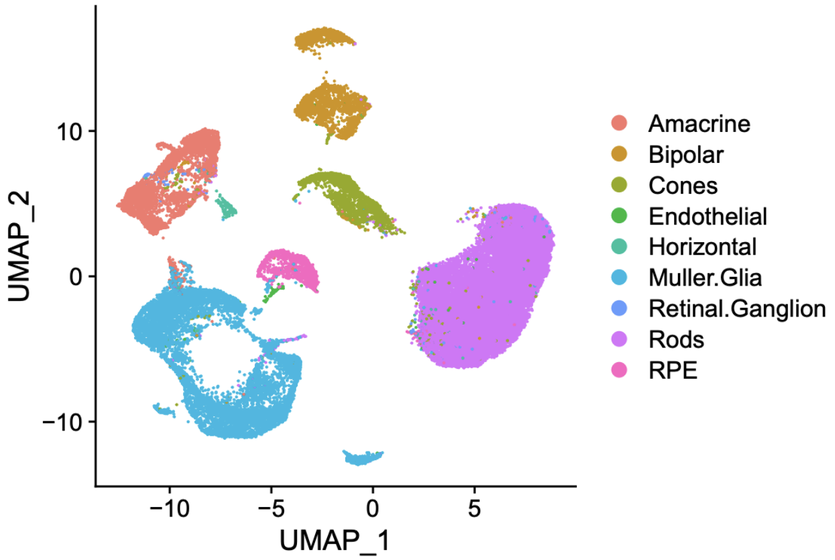
Elbow plot



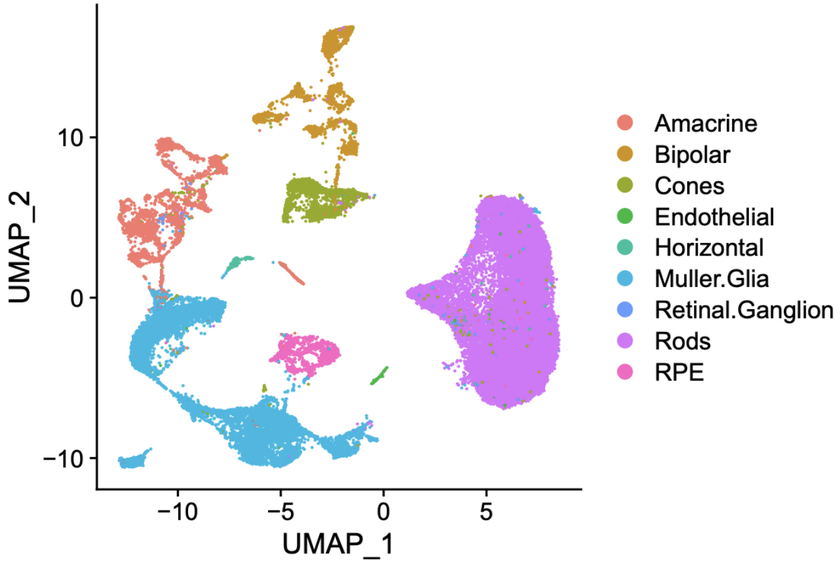
of PCs = 5



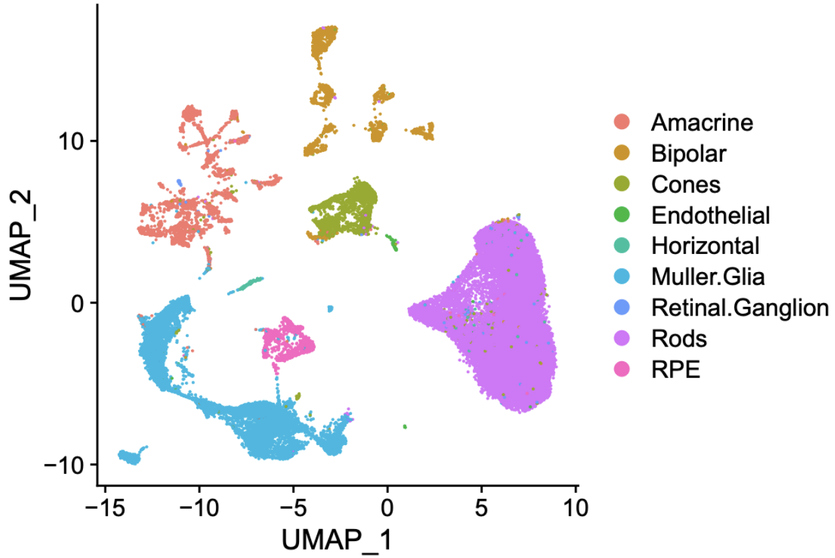
of PCs = 10



of PCs = 30

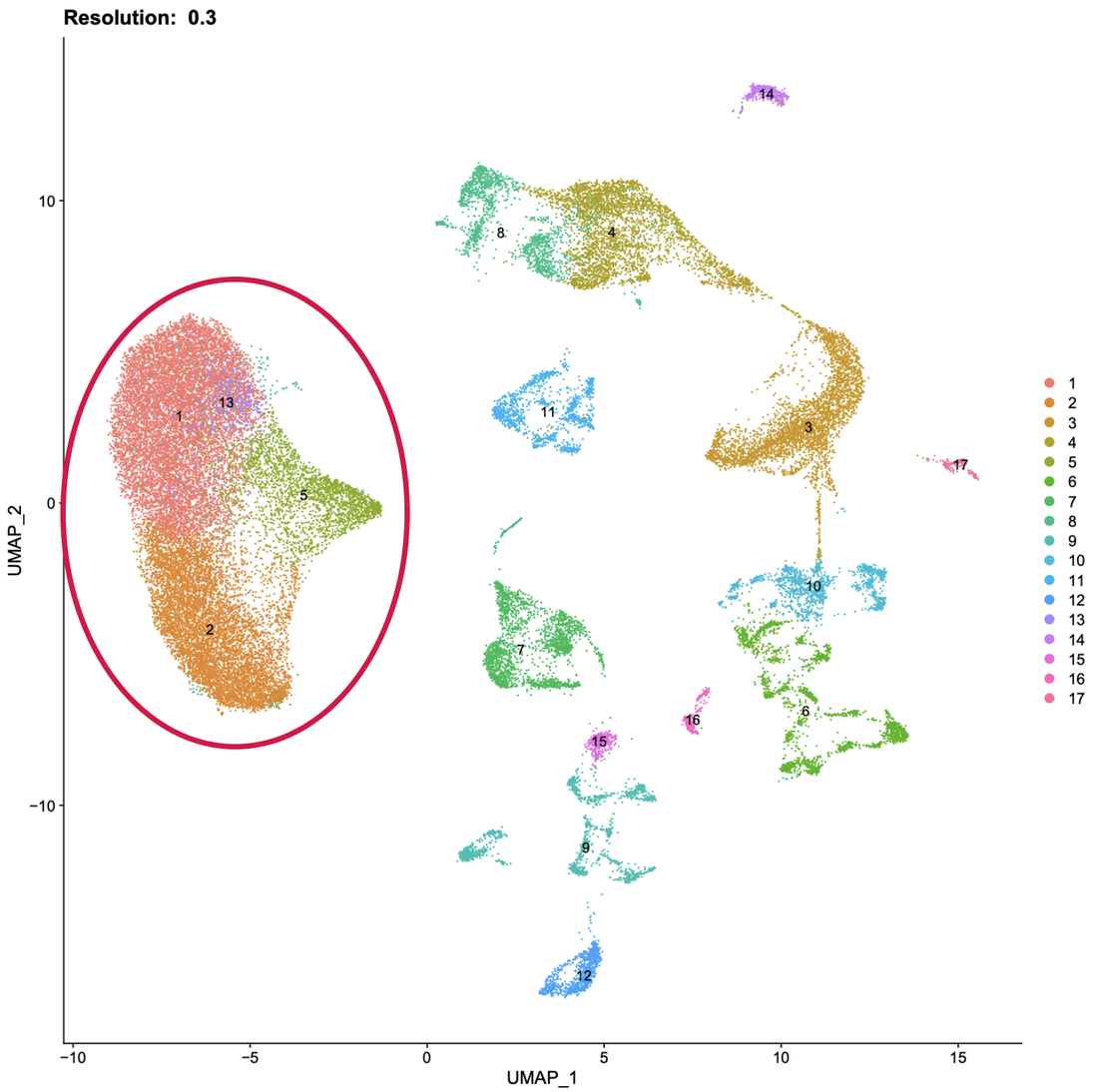


of PCs = 50



Clustering

- Generating clusters is common next step after PCA
- Two steps in Seurat
  - FindNeighbors() – uses PCs, determines overlap in close cells
  - FindClusters() – uses FindNeighbors() similarity scores, groups cells optimally
- Important parameter for FindClusters() is resolution
- Resolution determines how broad or fine clustering is
  - Too low, group heterogenous cells together
  - Too high, split homogenous groups arbitrarily


Resolution: 0.3
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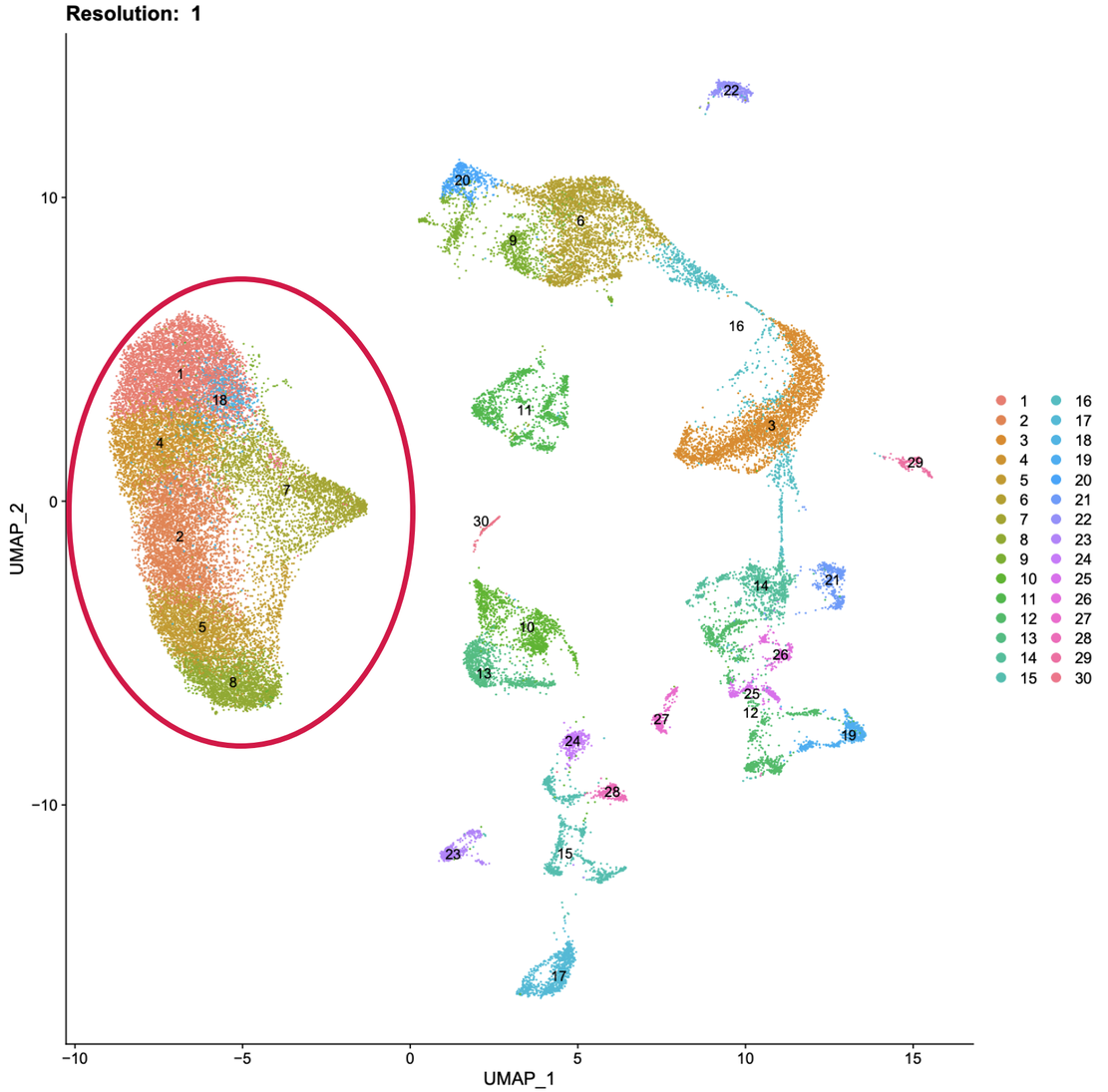
Resolution: 1
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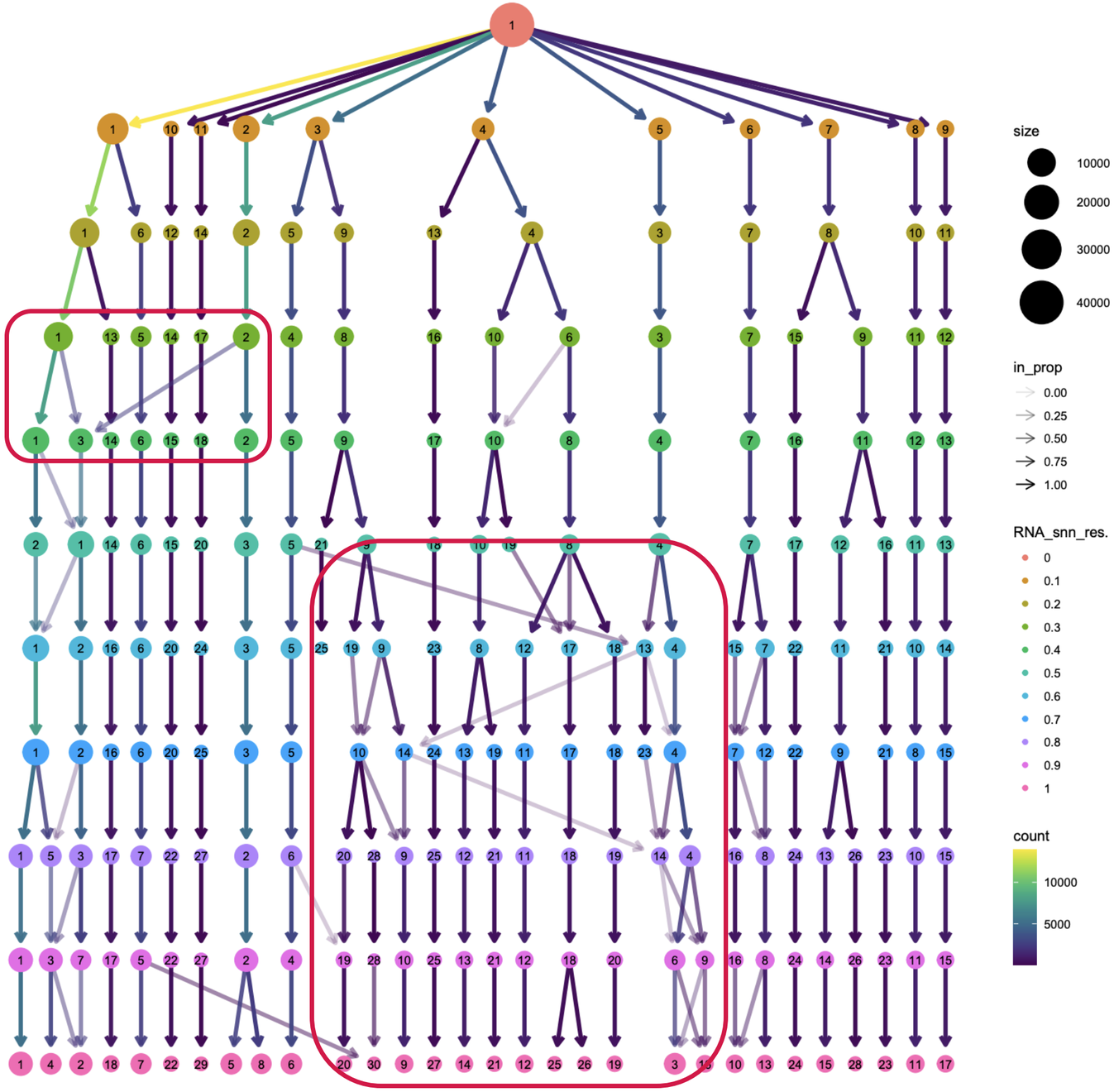
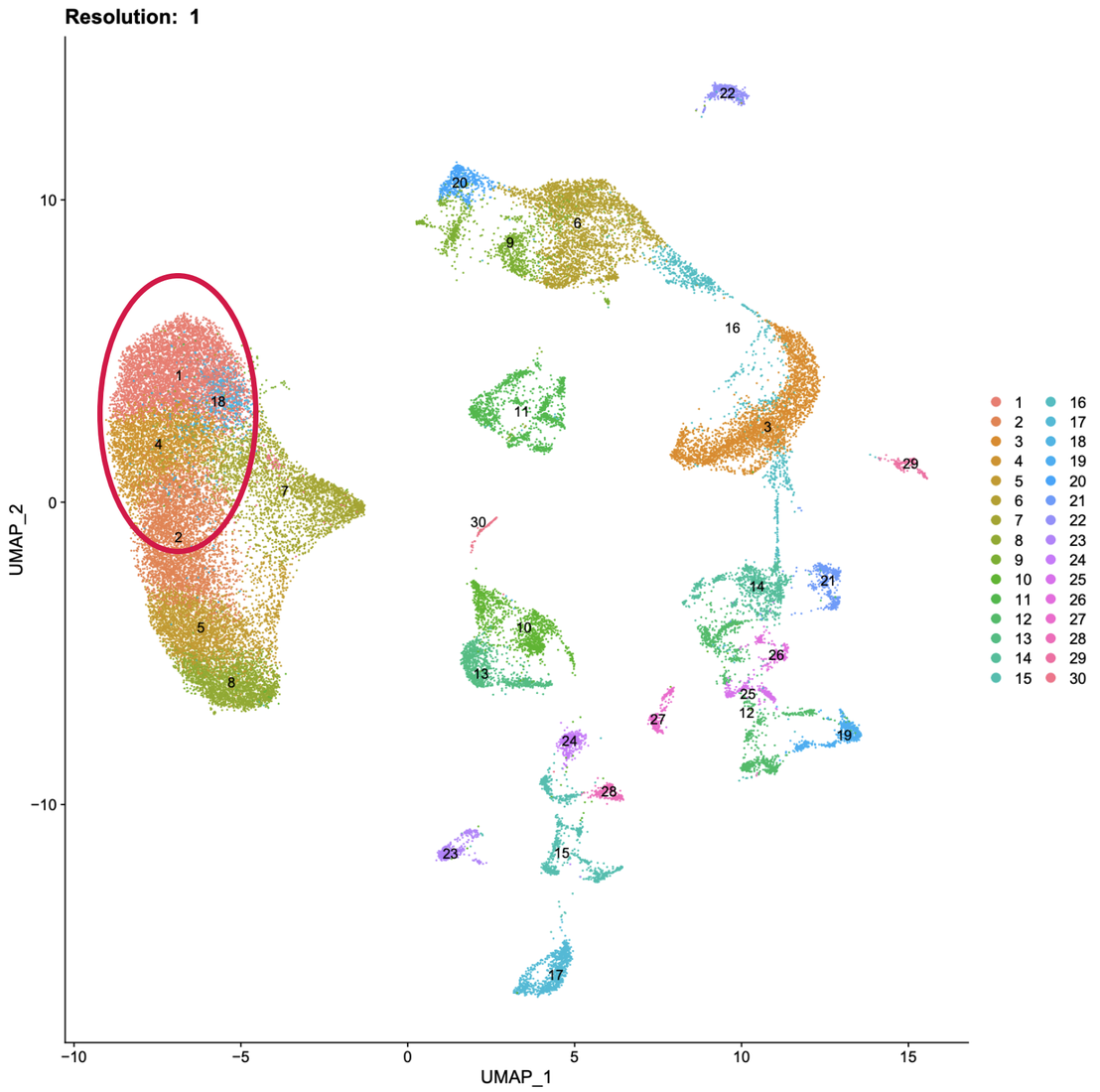
- Each circle is a cluster.
  - Size represents relative number of cells
- Each level corresponds to resolution.
  - Resolutions 0 to 1, increments of 0.1
- Arrows represent how cells split, move across clusters from one resolution to the next
- Multiple incoming arrows to cluster, cells jumping, not splitting suggest overclustering




Resolution: 1
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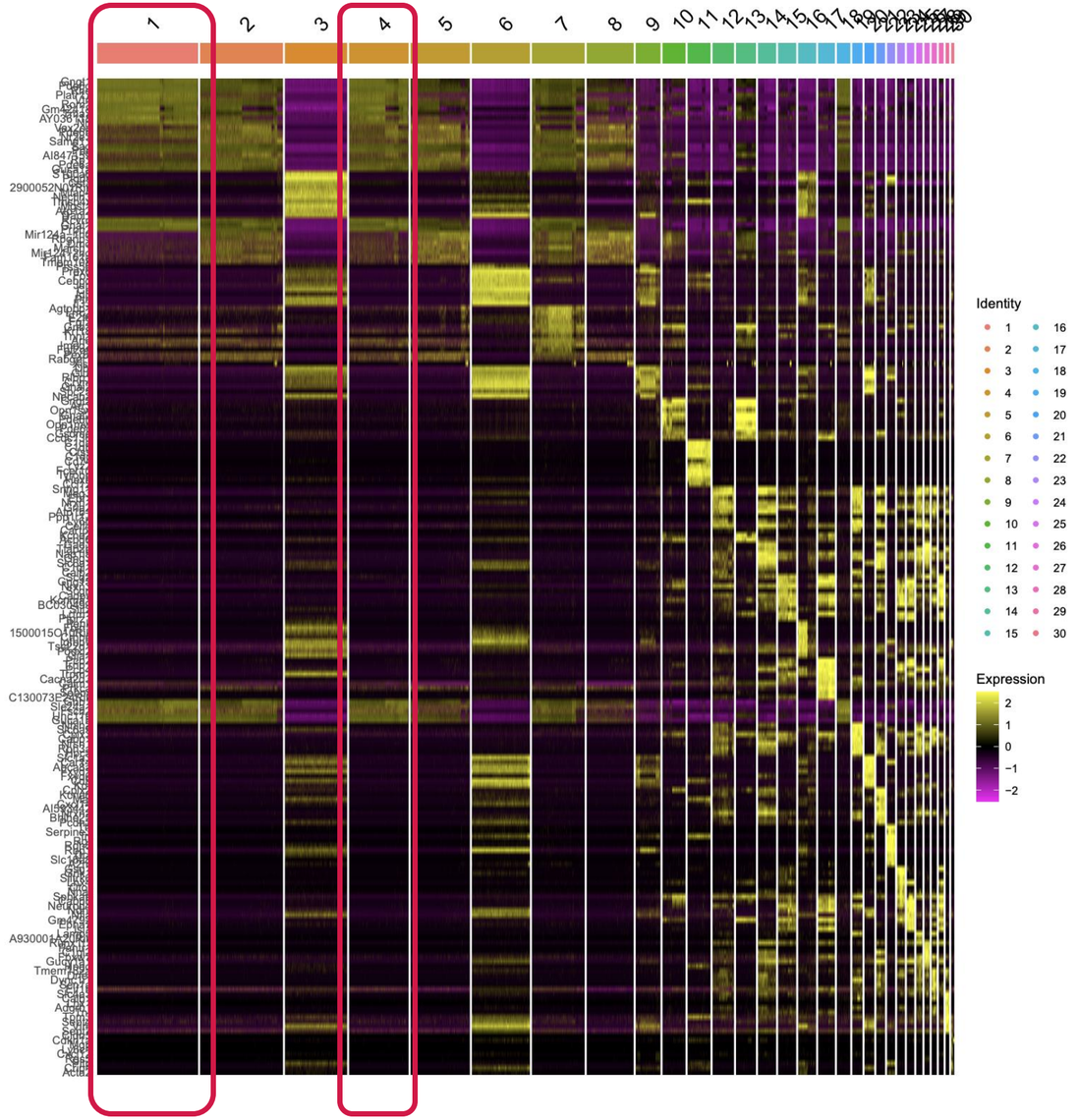
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Non-linear dimensionality reduction and visualization

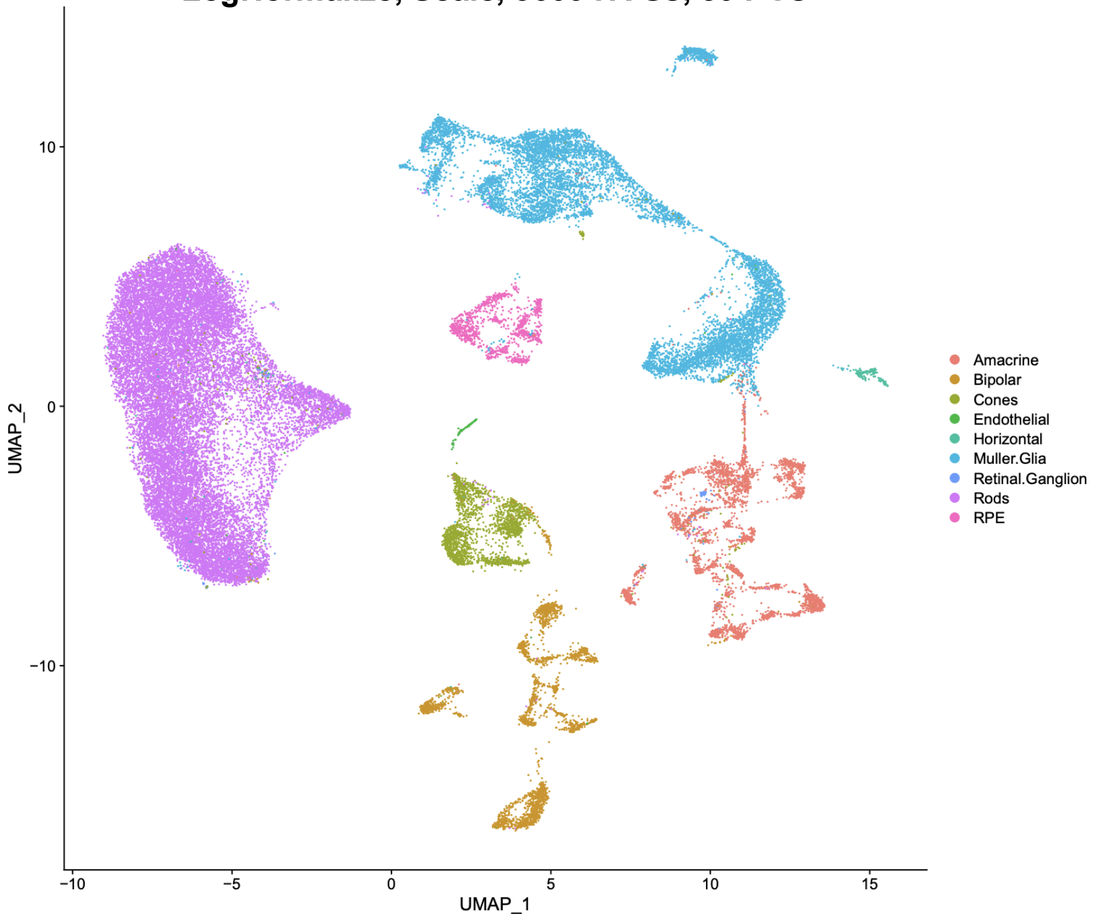
- Non-linear dimensionality reduction for visualization common
  - UMAP
- Display cells in two dimensions, preserve underlying data structure
  - E.g clusters ideally group in UMAP
- Uses PCs, improves speed, loses some structure
- Non-linear dimensionality reduction methods are imperfect.
- 2D cannot capture full picture of true structure

Non-linear dimensionality reduction and visualization

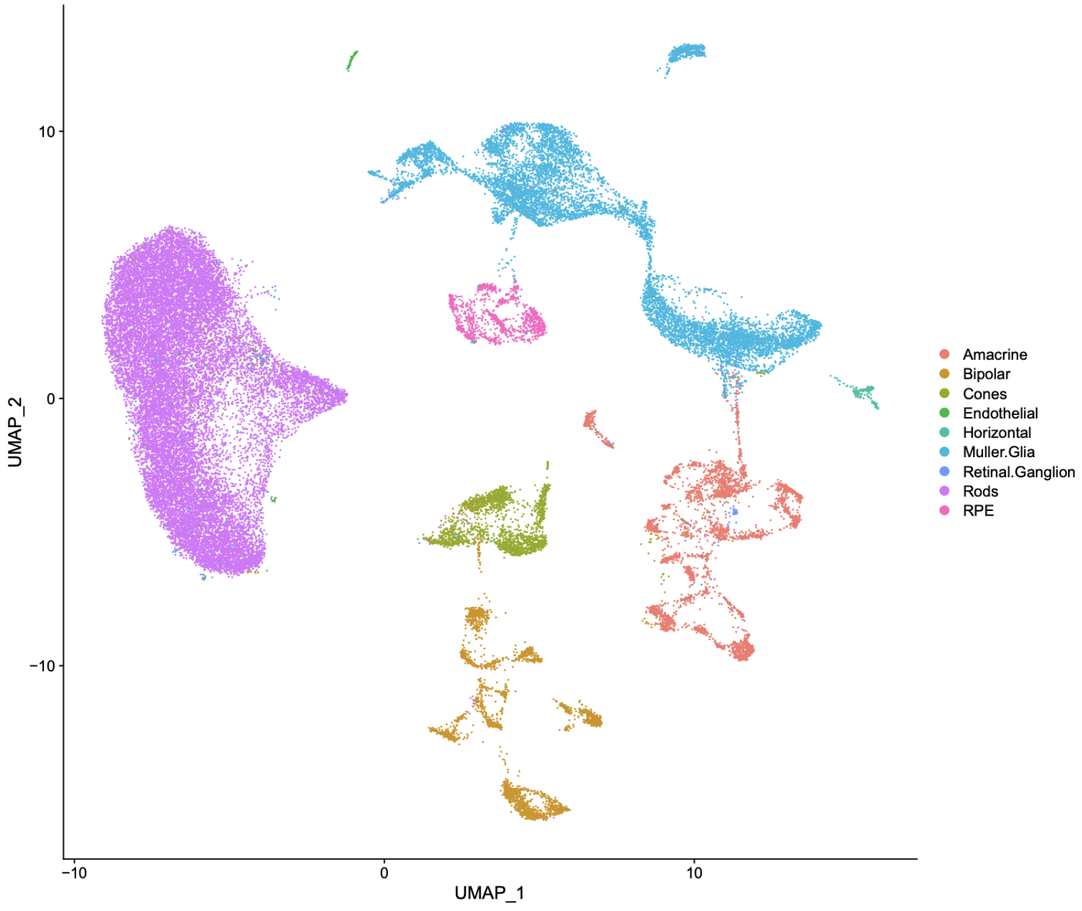
- Visualizations can help you refine data, e.g. patterns may suggest:
  - Ambient RNA contamination, doublets
  - Cells grouping on cell cycle
  - Cells grouping on filtering metrics
- Do not rely on UMAPs for biological interpretation.
- Shape can easily change based on numerous factors
  - Seed, normalization, scaling, variable features, principal components
- Structure may match biology, but verify through additional methods
- Don’t force structure to fit biology

Non-linear dimensionality reduction and visualization

**“Standard” processing –
LogNormalize, Scale, 3000 HVGs, 30 PCs**

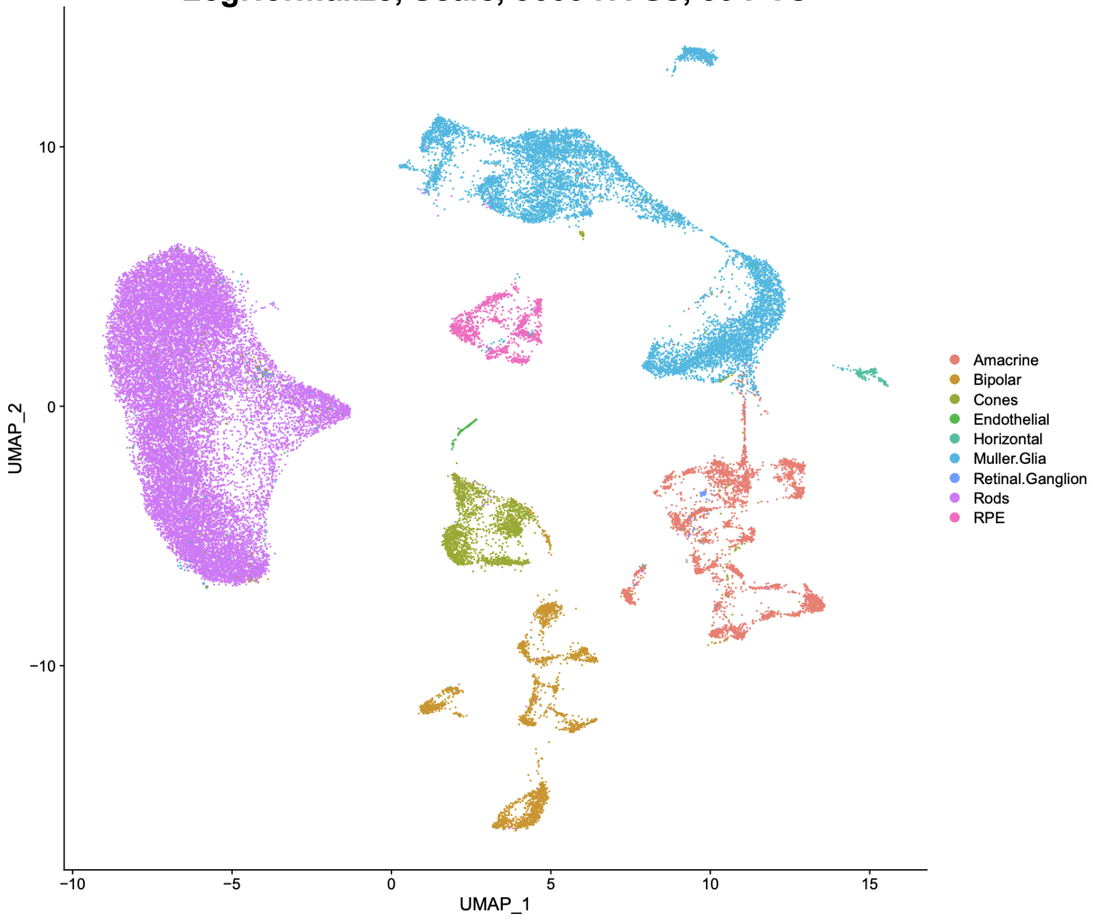


**“Standard” processing –
different seed value (22465)**

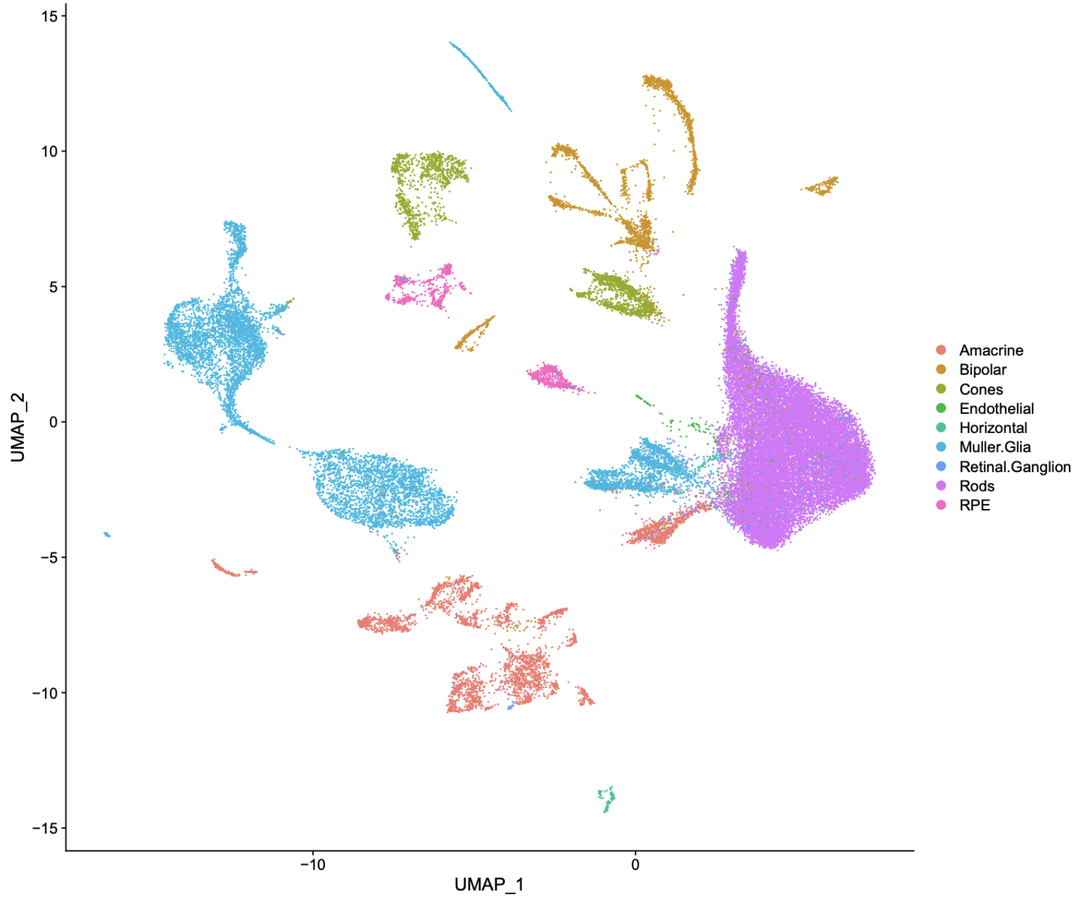


Non-linear dimensionality reduction and visualization

“Standard” processing –
LogNormalize, Scale, 3000 HVGs, 30 PCs

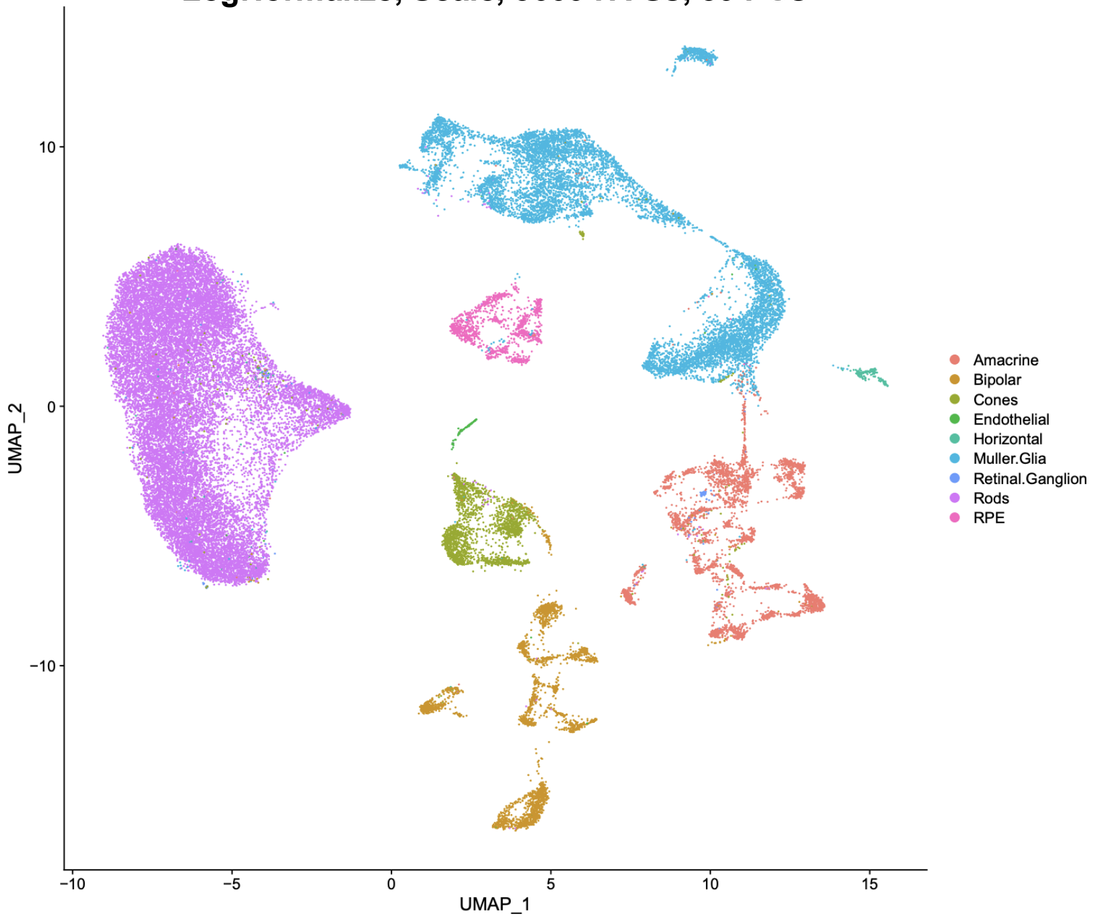


Un-normalized

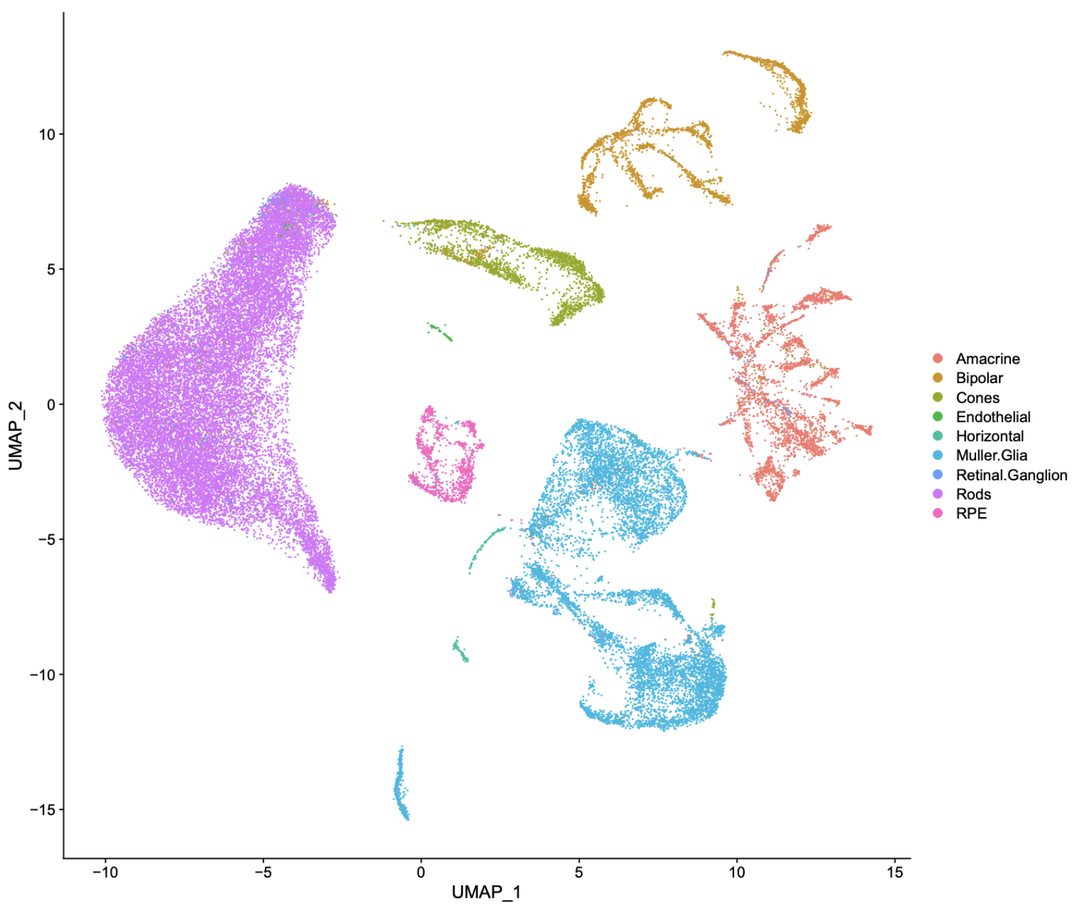


Non-linear dimensionality reduction and visualization

“Standard” processing –
LogNormalize, Scale, 3000 HVGs, 30 PCs

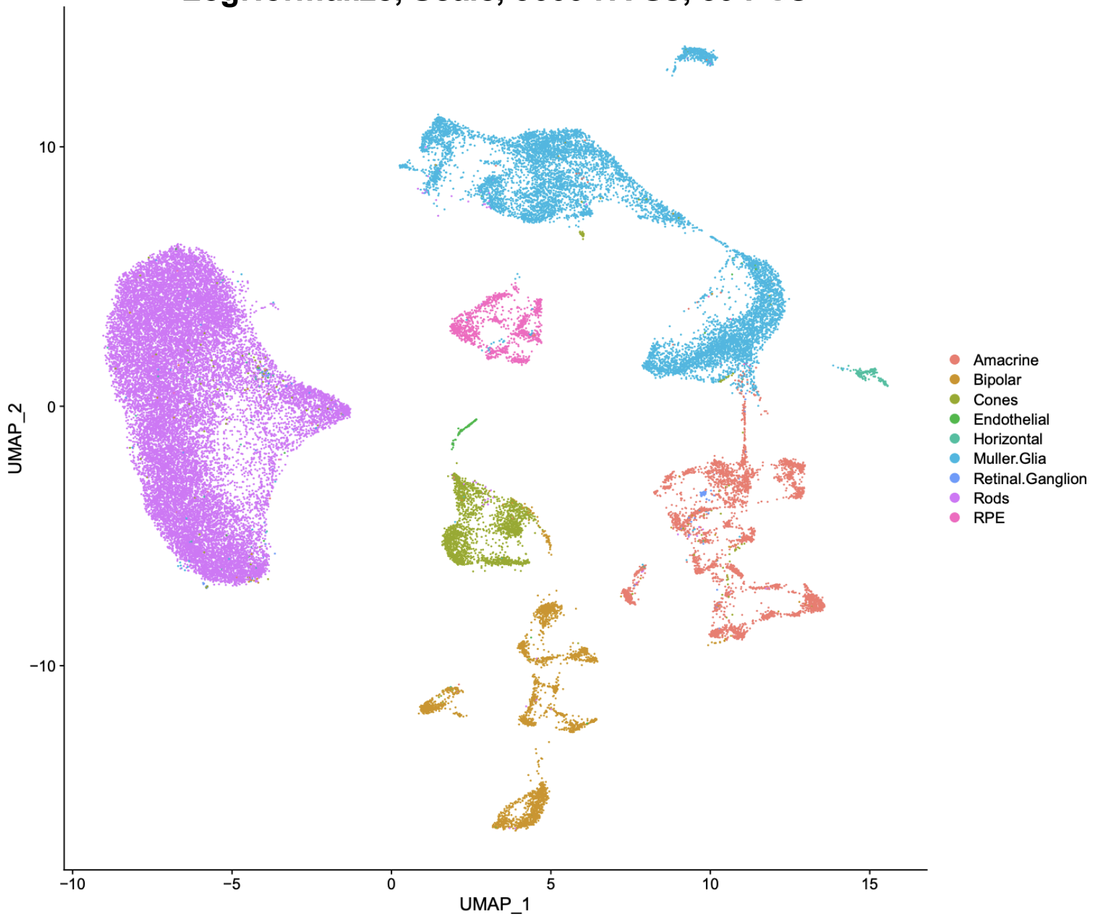


CLR Normalization

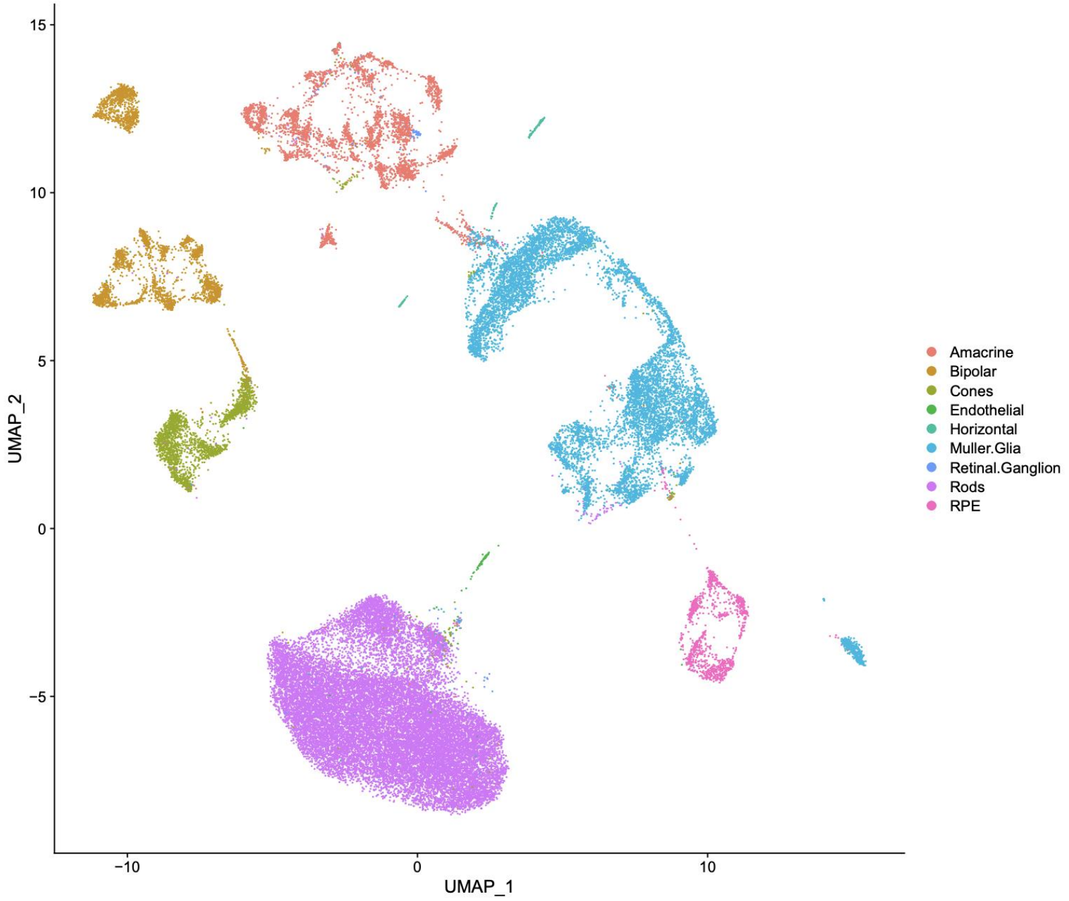


Non-linear dimensionality reduction and visualization

“Standard” processing –
LogNormalize, Scale, 3000 HVGs, 30 PCs

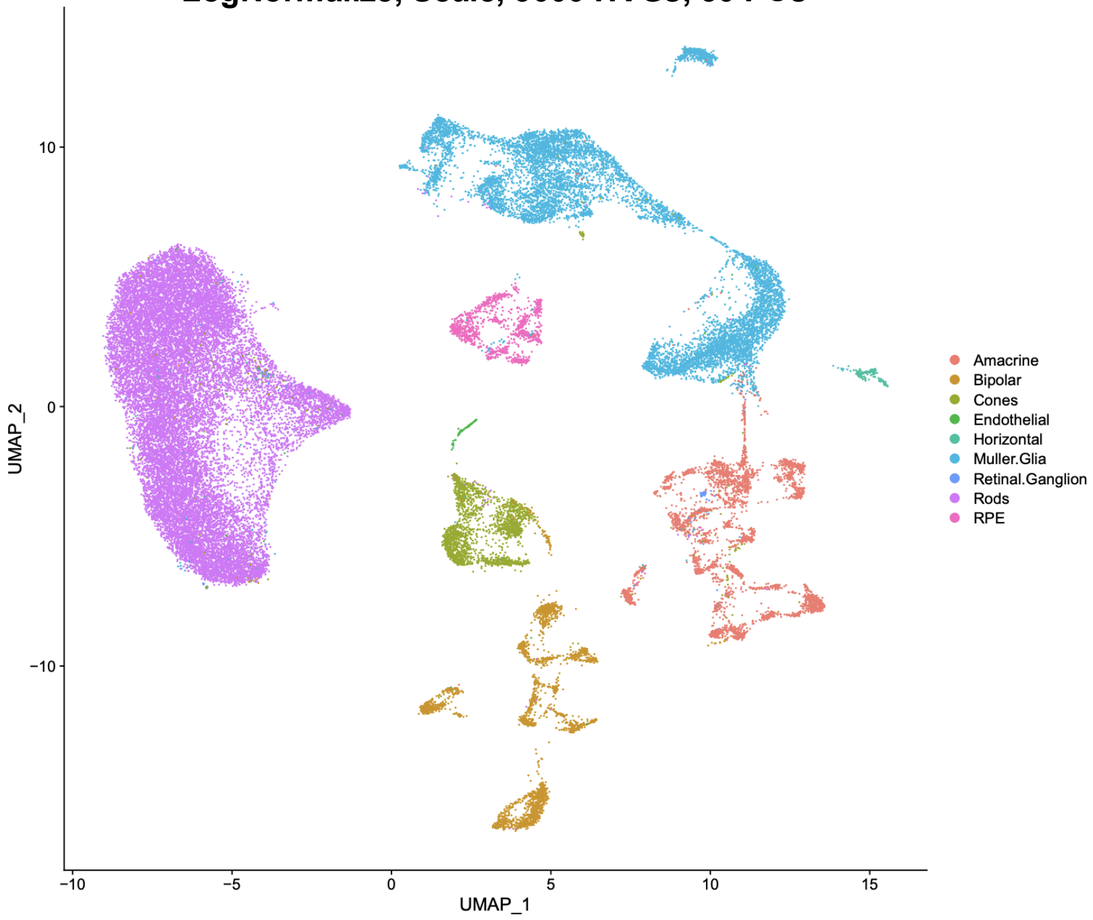


Un-scaled

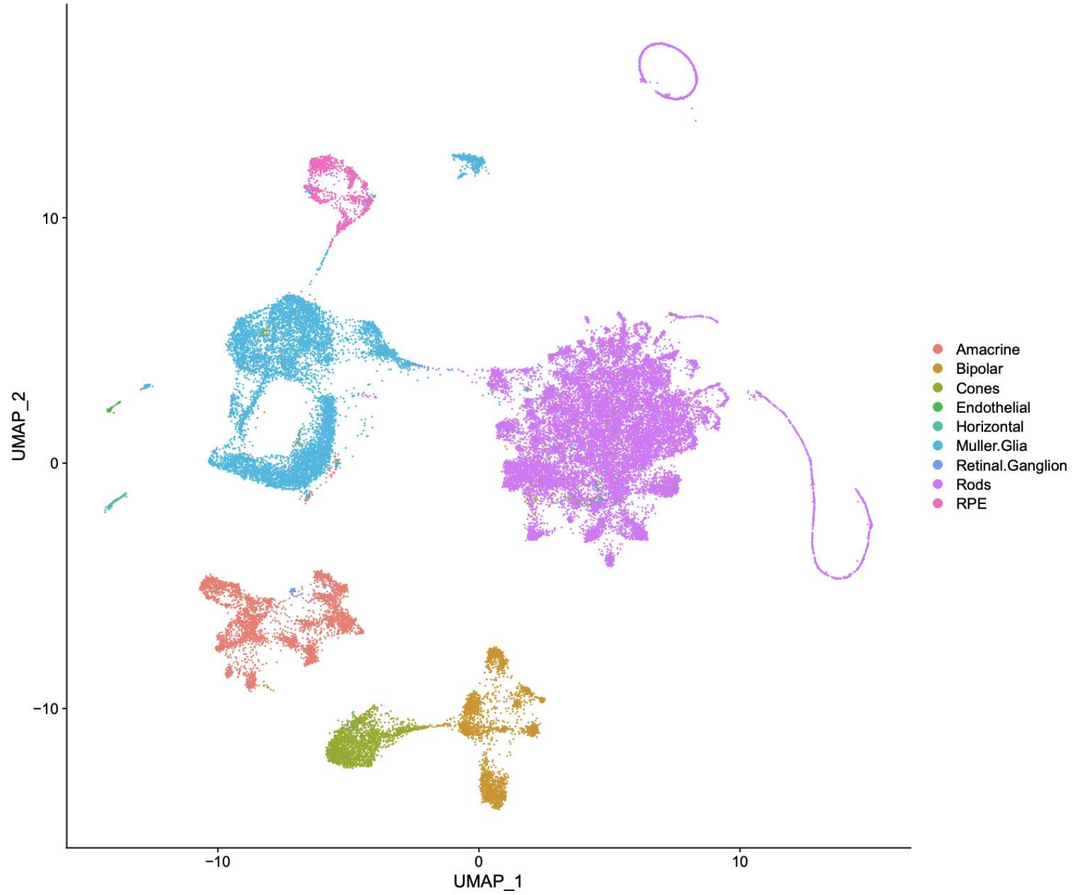


Non-linear dimensionality reduction and visualization

“Standard” processing –
LogNormalize, Scale, 3000 HVGs, 30 PCs

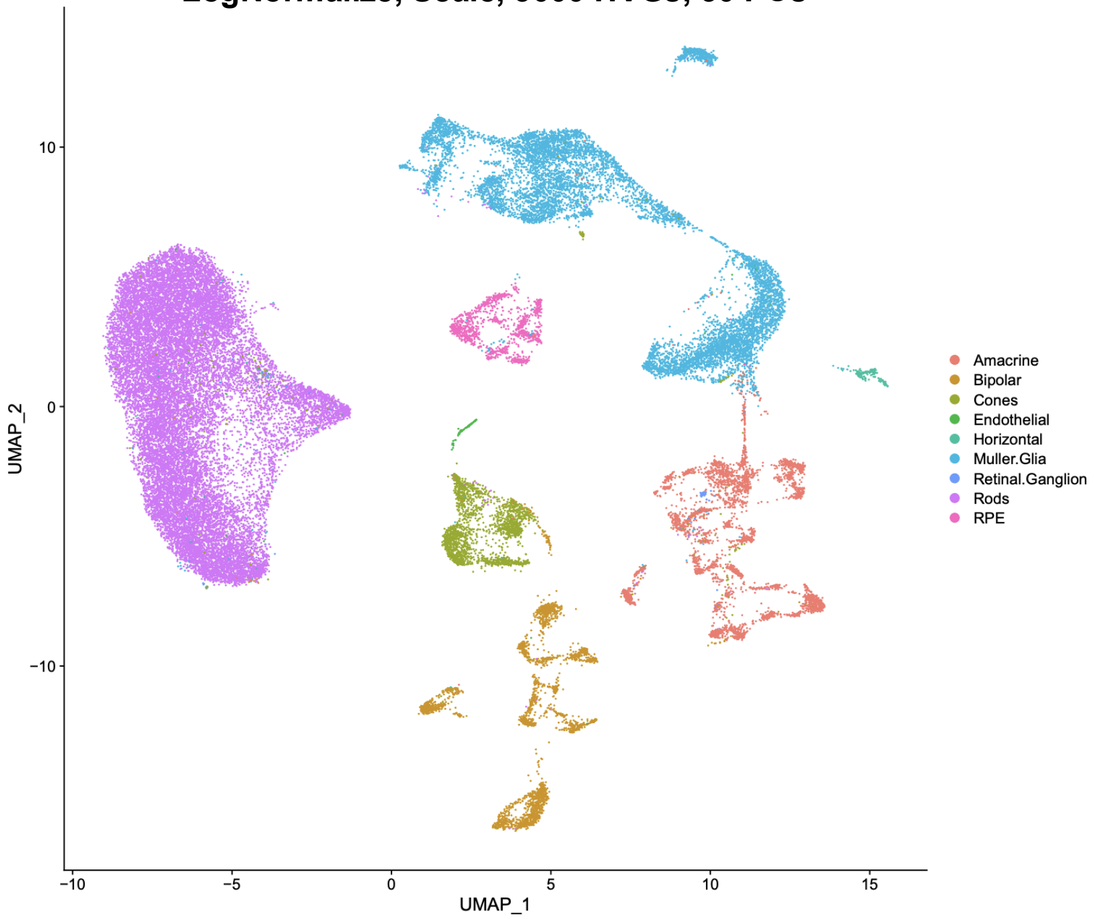


500 HVGs

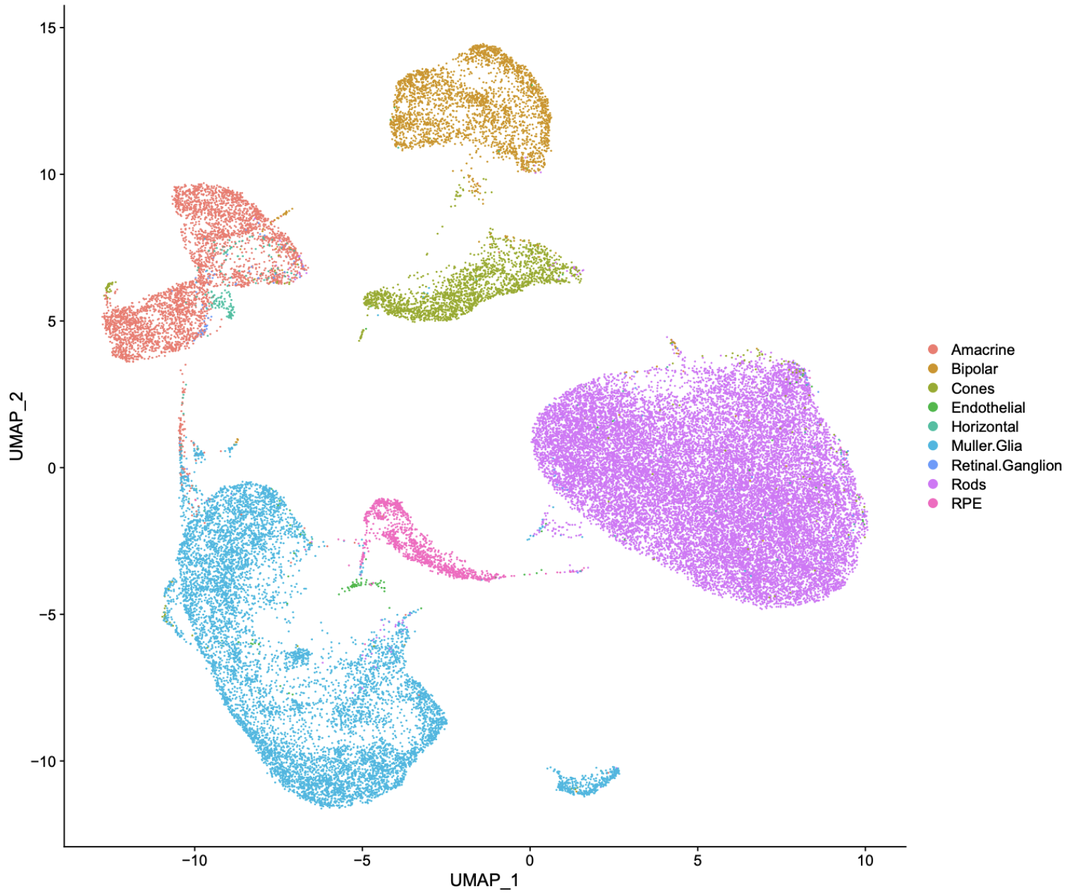


Non-linear dimensionality reduction and visualization

“Standard” processing –
LogNormalize, Scale, 3000 HVGs, 30 PCs

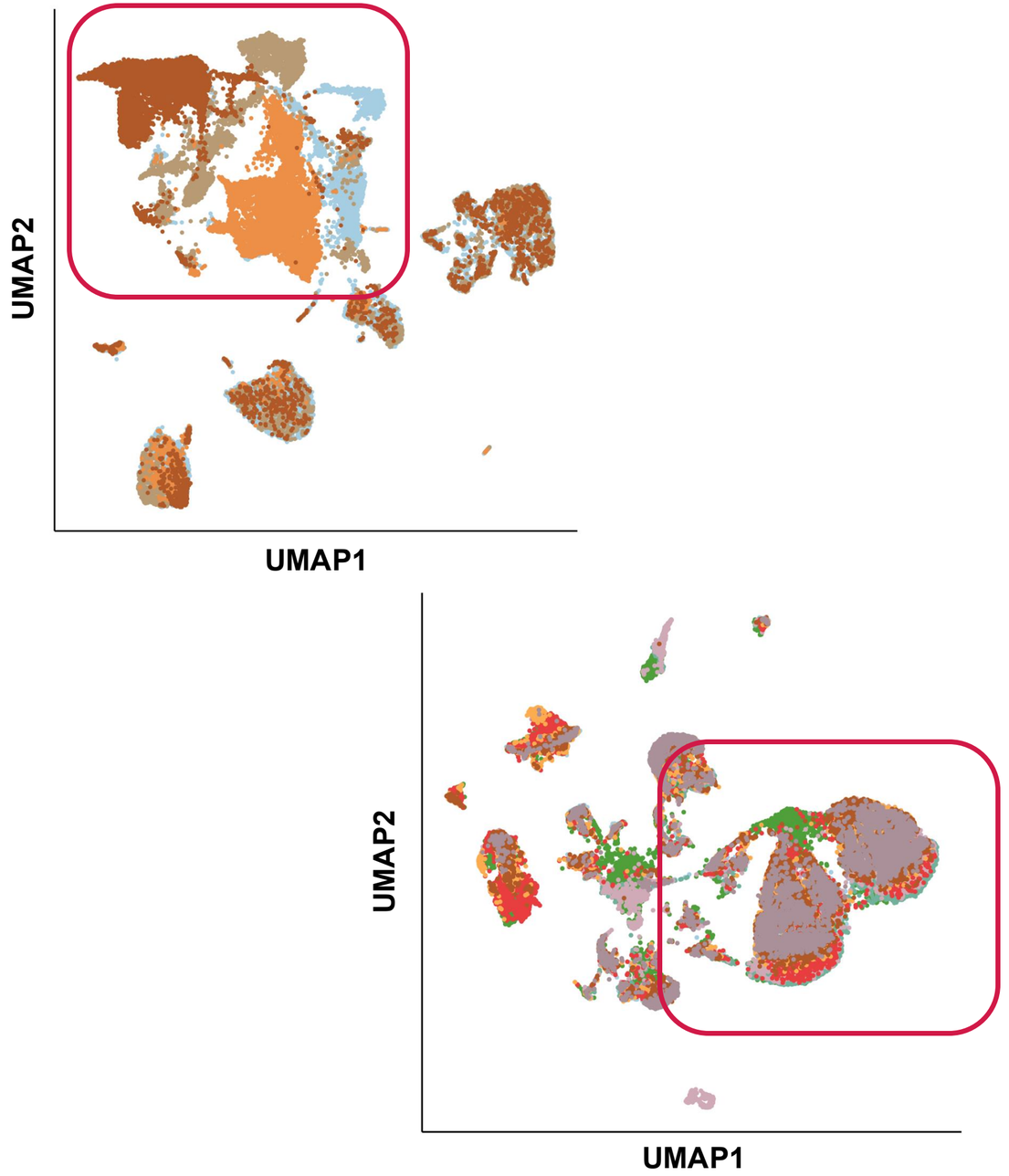


7 PCs



Integration

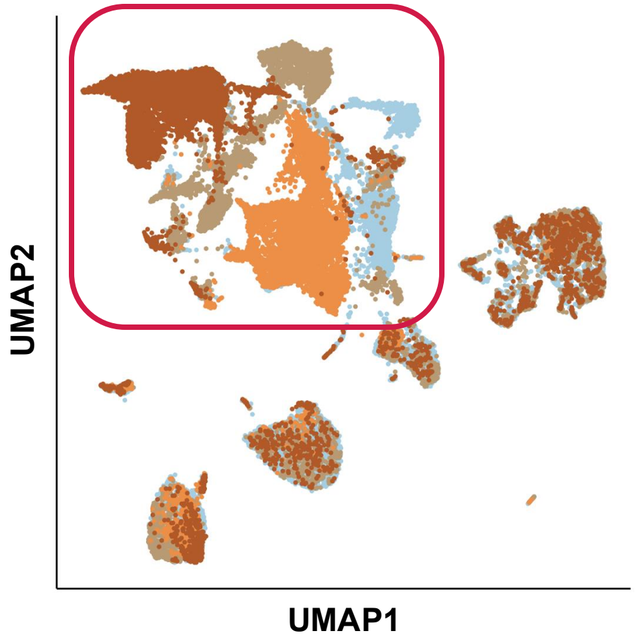
- Often group multiple samples for clustering, visualization, downstream analysis
- Simple merges show sample-based batch effects
  - Distinct cluster per sample
  - Rainbow effect within clusters

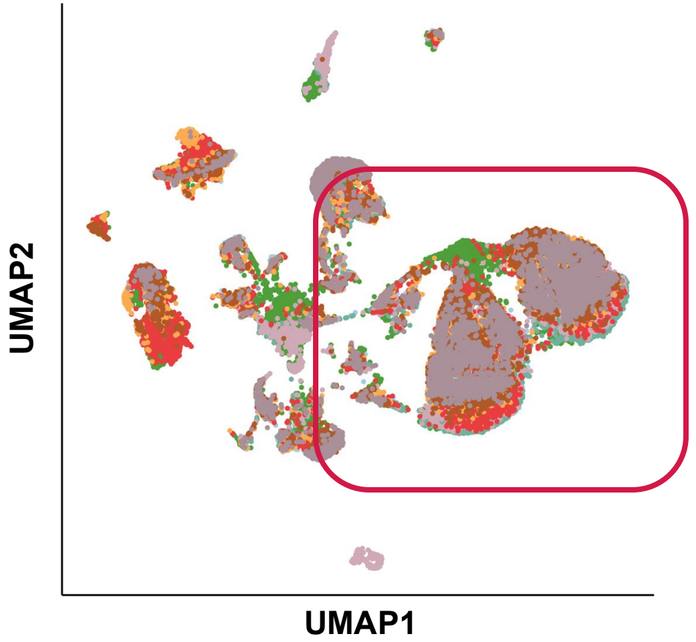


Integration

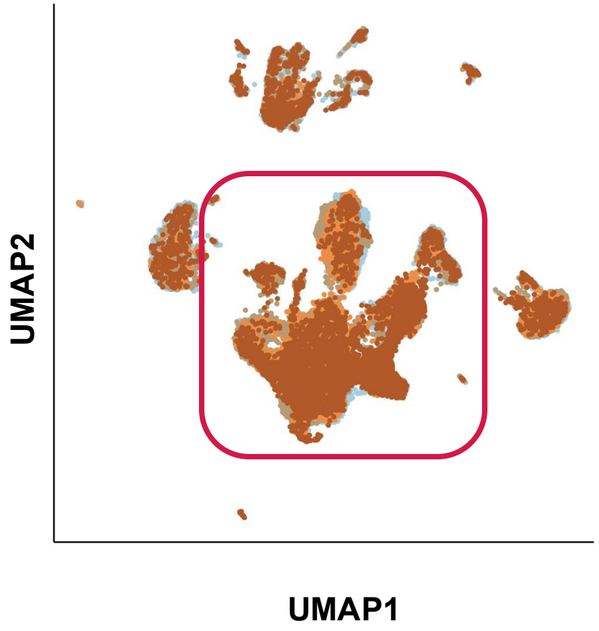
- Many tools, methods for integration
- Bioinformatics core uses three methods
  - Harmony (separate package)
  - LIGER (separate package)
  - Anchor-based integration (implemented in Seurat)
- Harmony attempts to reduce technical variation, maintain biological variation
- LIGER assumes batch effects are technical
- Step-wise anchor-based integration saves resources, can introduce bias
- No consensus on “best” method, depends on size, complexity, etc.

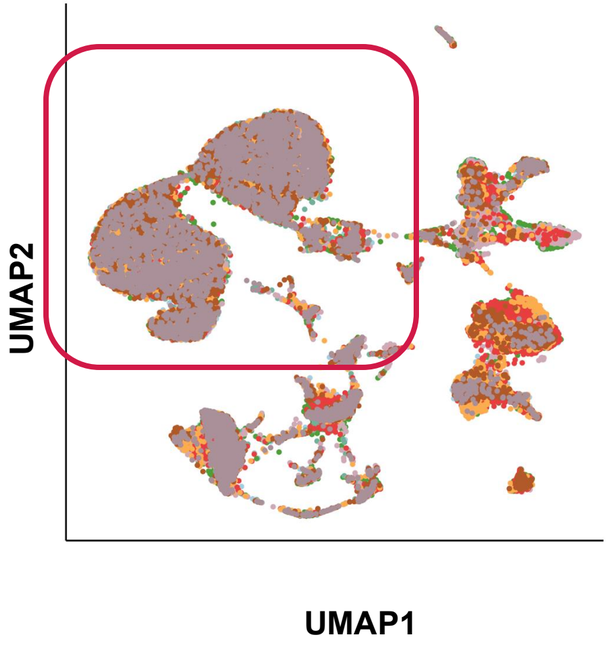
Non-integrated:





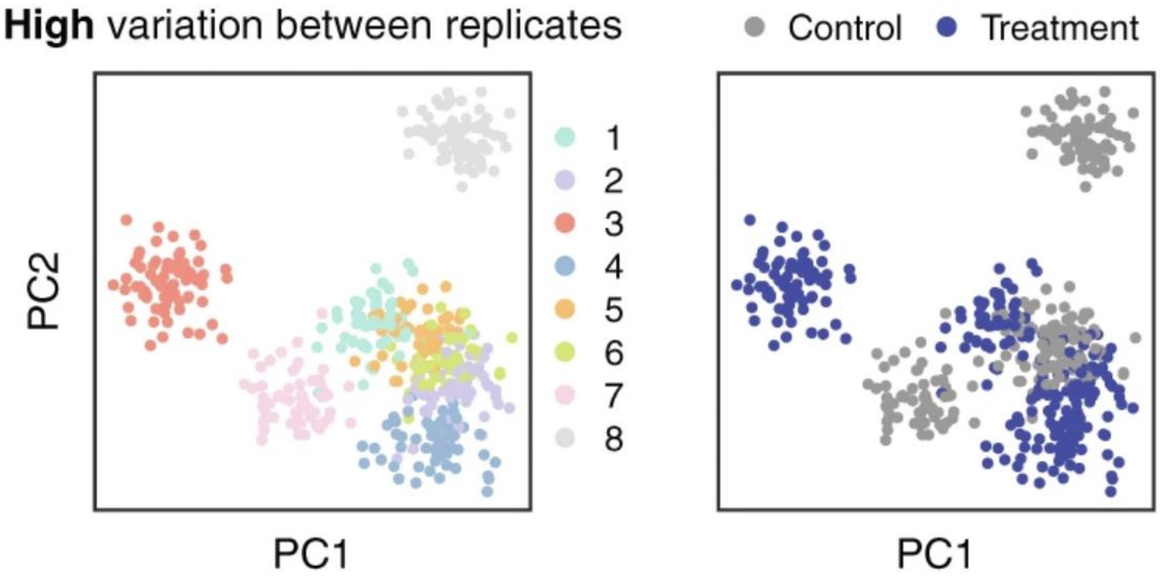
Integrated:





Differential Expression Analysis

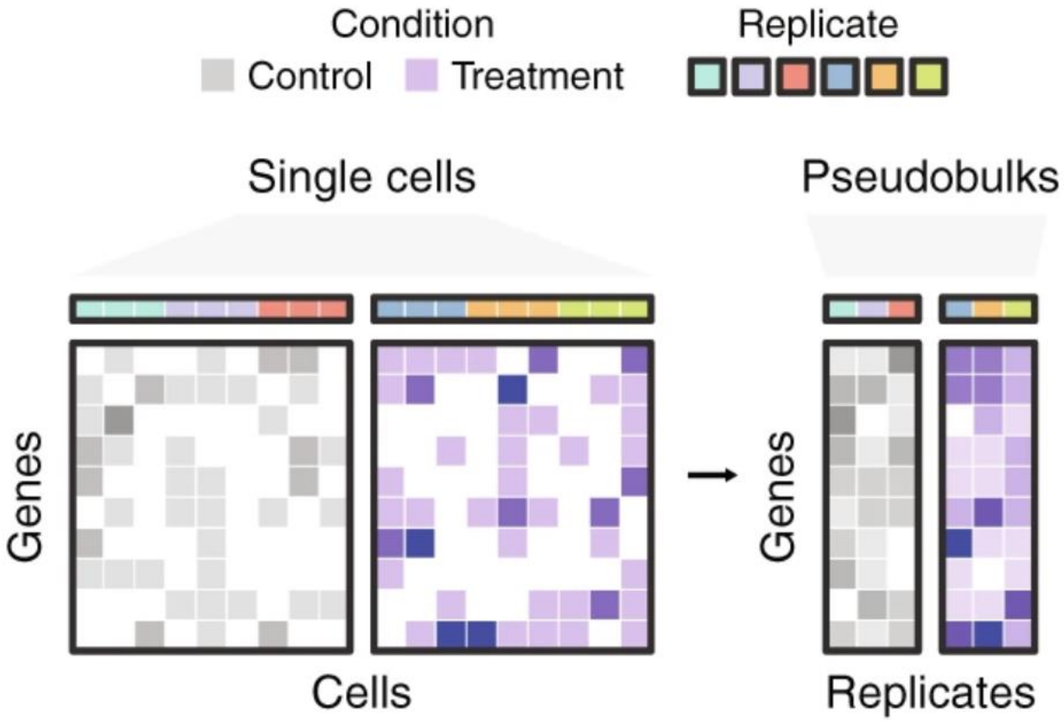
- Consider batch effects when running, interpreting DE
  - Technical and biological effects
  - Dropout, inflated zero counts
  - High variability across samples



Squair (2021). Nature Communications.

Differential Expression Analysis

- Two categories for methods:
  - Cell-level
  - Pseudobulk
- Cell-level methods treat cell expression profiles individually.
  - e.g. Wilcoxon rank sum (Seurat's default)
- Pseudobulk methods aggregate expression profiles per sample.
  - e.g. DESeq2 (bulk RNA-seq method)



Squair (2021). Nature Communications.

Differential Expression Analysis

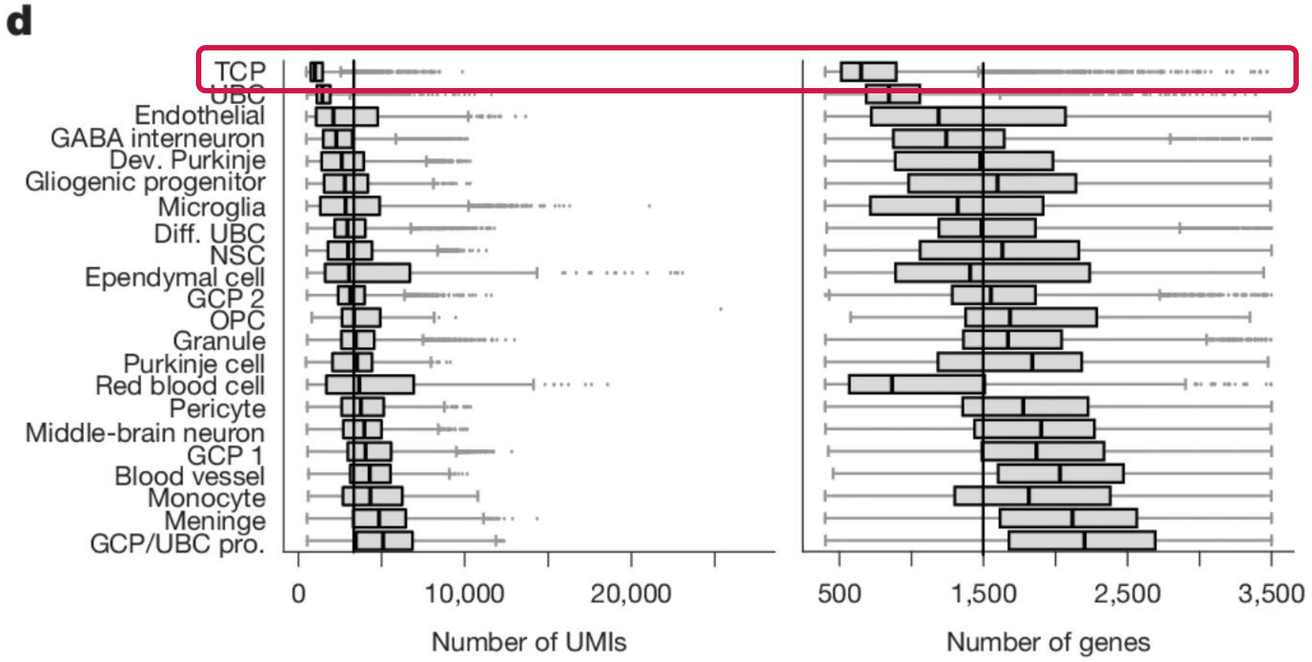
- Cell-level methods prone certain issues
  - Inflated significance of p-values
  - Inflated number of false positives
- Fail to address dependence of cells in same sample
- Still useful for broad results
  - e.g. generating per cluster markers

Differential Expression Analysis

- Pseudobulk methods address certain issues
  - Reduce inflated significance
  - Reduce false positive rates
- Allow more complex models, confounding variables
- Need at least two, ideally three replicates per variable of interest
- Can still see batch effects, inflated significance, false positives
- Reproduce results in other datasets
- Confirm via independent wet lab methods

Cell Type Annotation

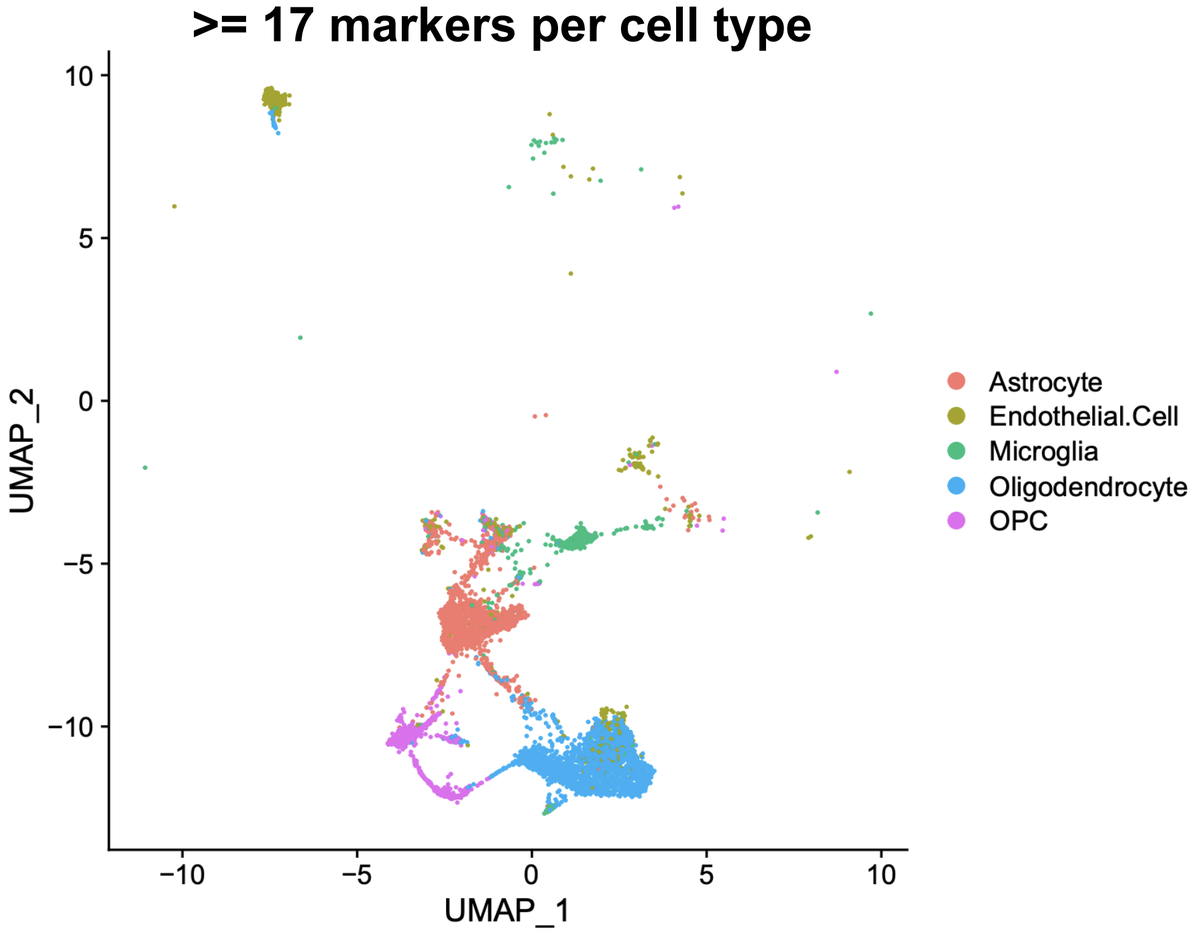
- Pitfalls upstream can lead to inaccurate annotations downstream
- Inaccurate annotations can lead to false conclusions

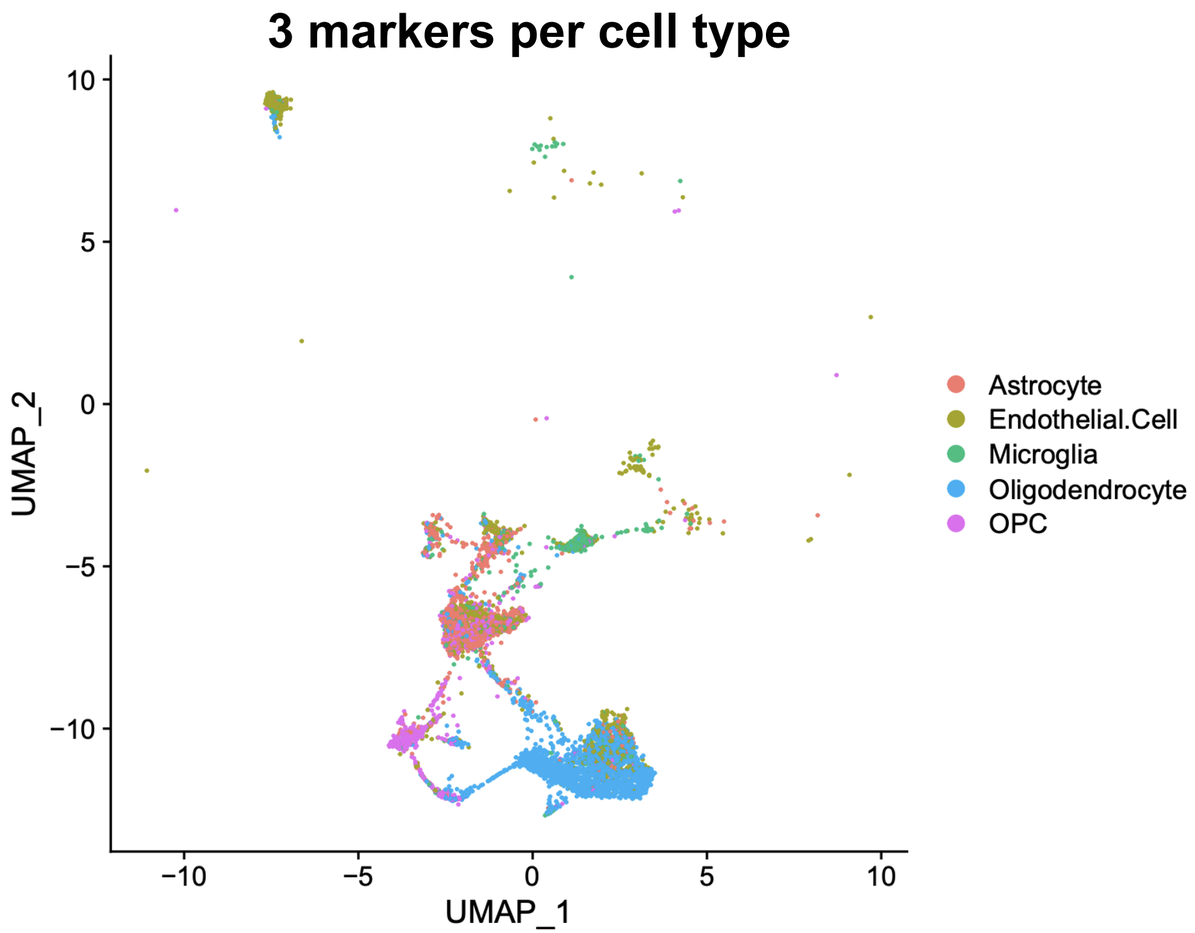


Smith (2025). Nature.

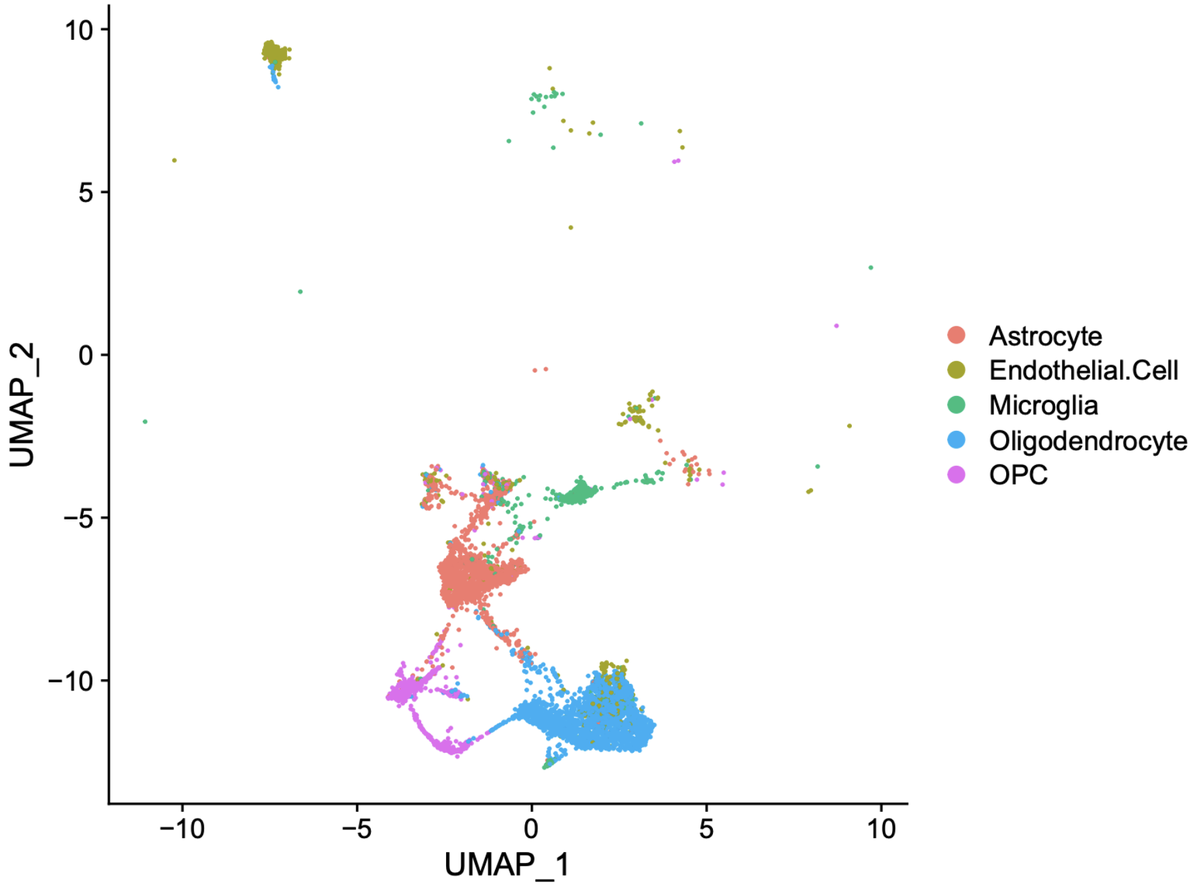
Cell Type Annotation

- Annotations can involve manual curation, semi-automated methods, fully automated methods, e.g.
  - Module scoring (semi-automated, uses curated gene marker lists)
  - SingleR annotation (automated, uses annotated single cell or bulk references, generates broad or fine labels)
  - Reference label transfer (automated, uses annotated single cell objects)
- Consider number of cell type markers use for module scoring
  - Too few, overlapping markers can cause mislabeling, miss subtypes
  - More makers reduces noise in annotations
- Module scoring will force label assignment
  - Can only assign labels provided as input

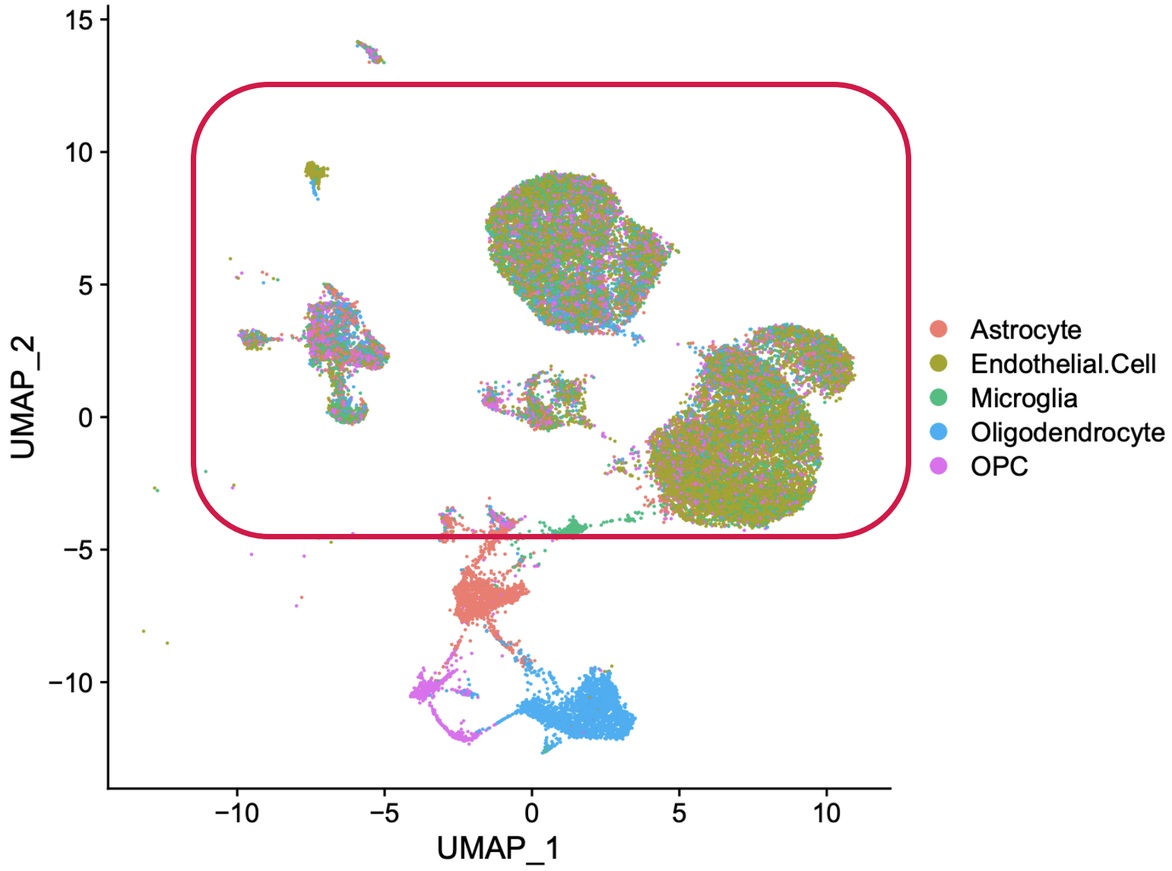

>= 17 markers per cell type
UMAP_2
UMAP_1
10
5
0
−5
−10
−10
−5
0
5
10
Astrocyte
Endothelial.Cell
Microglia
Oligodendrocyte
OPC


3 markers per cell type
UMAP_2
UMAP_1
10
5
0
−5
−10
−10
−5
0
5
10
Astrocyte
Endothelial.Cell
Microglia
Oligodendrocyte
OPC

Non-neuronal cells



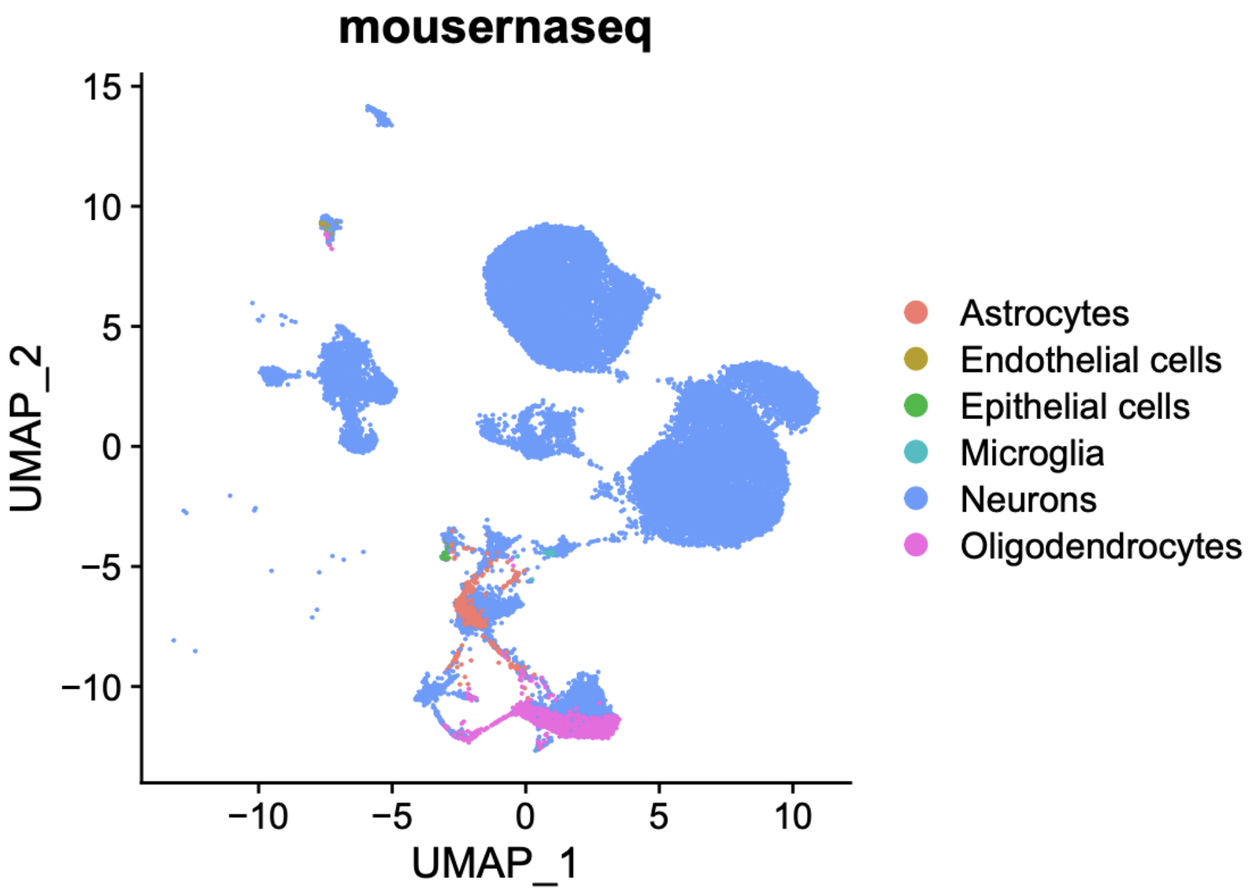
Neuronal cells

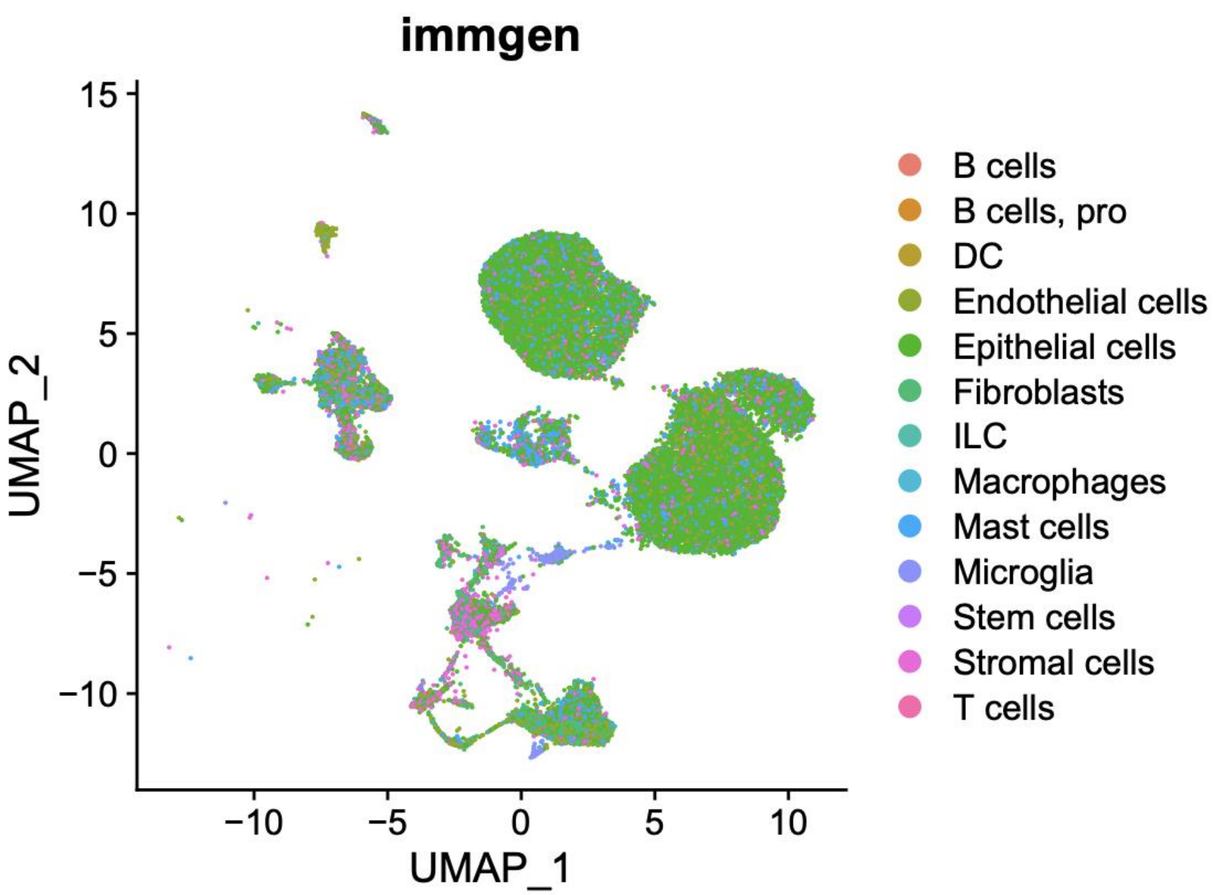


Cell Type Annotation

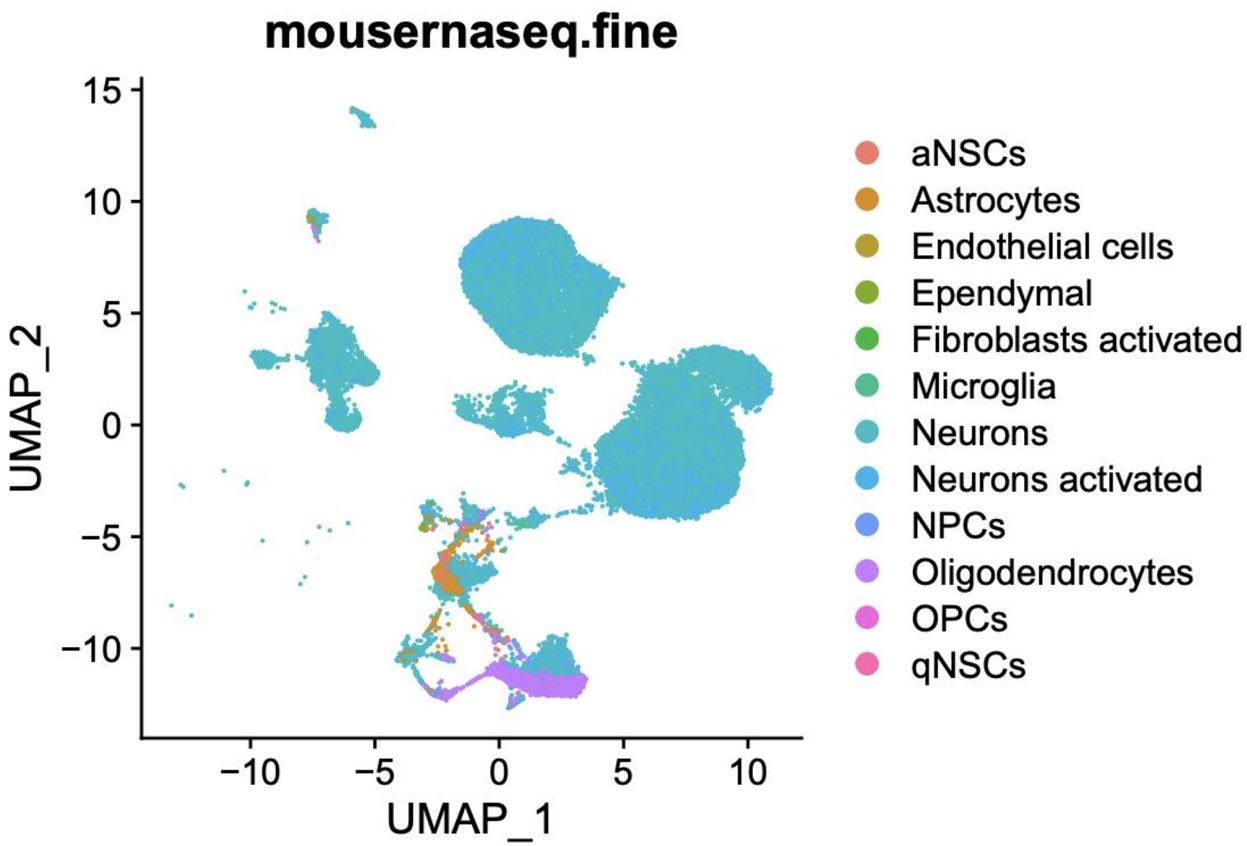
- Important to understand resources used for automated tools (e.g. SingleR, reference label transfer)
  - How are cell types annotated?
  - What cell types are present?
  - How detailed are cell type labels?
- Do methods force labels to be assigned?
- SingleR provides broad, fine labels, can prune cells
- Label transfer detail depends on reference, labels every cell
- Rely on combo of semi-automated methods, fully automated methods, manual annotation

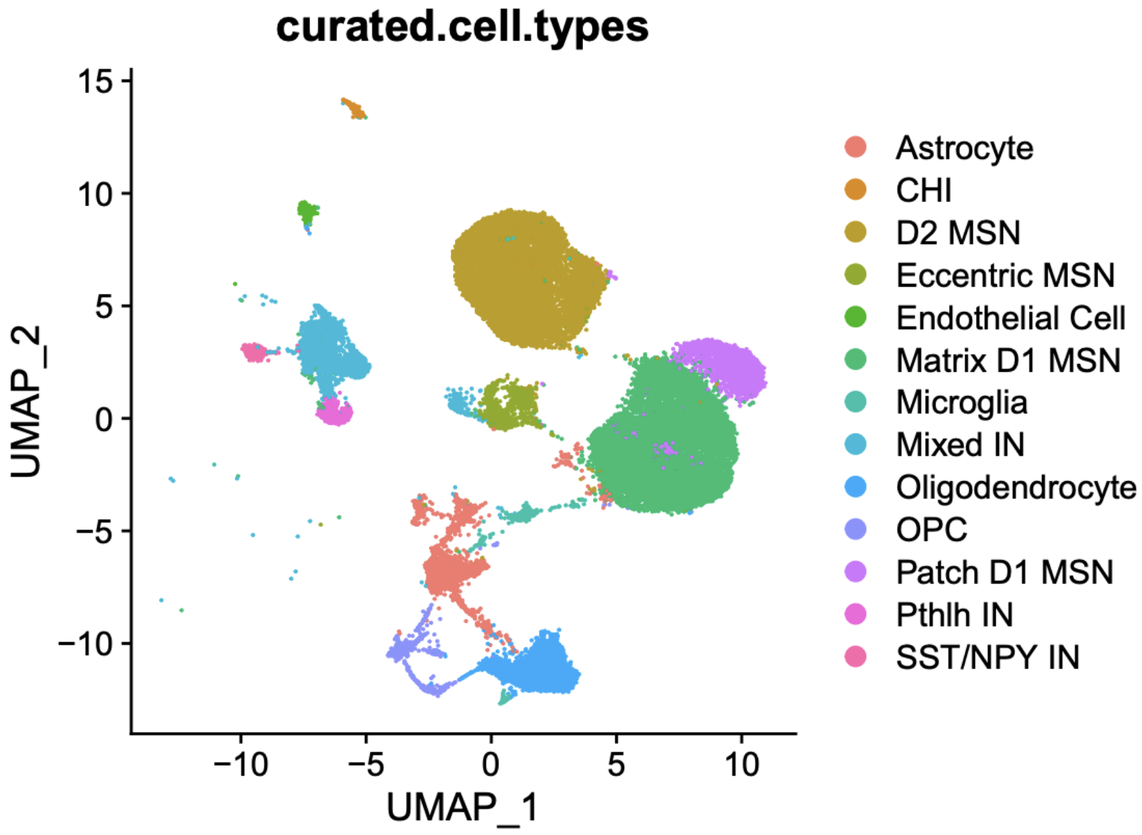
Cell Type Annotation





Cell Type Annotation





Summary

- Pitfalls can occur during most steps.
- Analysis often includes redoing, refining steps
- These pitfalls can give you a starting point for fine tuning.
- Be careful not to overinterpret data
- Be careful not to overly adjust to fit expected biology
- Verify findings with additional methods
- Gold standard is wet lab verification

Our Tools & Resources

Tool/Resource	Description	Icon
Snap	sc/snRNA-Seq 10x; supports human, mouse, and dual genomes	
Snap-10x-Flex	sc/snRNA-Seq 10x Flex; supports human and mouse genomes	
sc-epigenie	scATAC-Seq 10x Flex; supports human and mouse genomes	
sc-atac-seq-bed-builder	Generate blacklist/promoter/enhancer BED files for analysis	
DevOps Containers	Reusable, independent container images for scRNA/ATAC-seq workflows	
Trainings & Workshops	Hands-on training courses and pipeline tutorials